INVESTIGATIONS INTO THE FACTORS WHICH INFLUENCE MEASUREMENTS DURING *IN VITRO* GAS PRODUCTION STUDIES

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DECLARATION

I declare that the work reported in this thesis is my own, and that the thesis is my own composition

signed

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ABSTRACT

Feed evaluation is an important prerequisite to the formulation of farm animal diets. Techniques for routine feed analysis must be rapid, simple to perform and must provide accurate, reliable results which are biologically meaningful. The aim of this thesis was to investigate the suitability and application of an *in vitro* gas production technique as a routine analytical tool in animal nutrition studies. Firstly, the effect of several biological, chemical and physical factors on the measurement of gas production were investigated. These factors included incubation temperature, head-space pressure, shaking movement of the *in vitro* cultures, the nature of the feedstuff (chemical composition, particle size and drying process), the source of the microbial inoculum and the apparatus used to measure gas production. In most studies, the manual pressure transducer technique of Theodorou *et al.* (1994) was used to investigate the effect of these factors. Incubation temperature, head-space pressure and shaking movement all had a significant effect on the amount of gas produced during the incubation of perennial ryegrass (*Lolium perenne*) hay. Of the four incubation temperatures investigated (25, 30, 39 and 45 °C), not surprisingly 39 °C was found to be the optimum temperature for incubation of batch cultures. Increasing the pressure in the head-space of the culture bottles (by increasing the interval at which gas was removed from the head-space from 2 to 4 or 6 h) led to a decrease in the amount of gas measured ($p < 0.05$), but did not alter dry matter (DM) loss or volatile fatty acid (VFA) production suggesting that the fermentation was not inhibited by the accumulation of gas. Continual shaking of the culture bottles throughout the incubation resulted in the detection of less gas than in those bottles which were not shaken throughout the incubation ($p < 0.01$). The nature and treatment of the substrate also affected gas production. Microwave, oven and freeze drying of perennial ryegrass resulted in higher cumulative gas production profiles than incubation of the fresh substrate ($p < 0.01$). Reducing feed particle size led to increases in cumulative gas production ($p < 0.01$). In the method of Theodorou *et al.* (1994) where fermentations were conducted in gas-tight sealed culture bottles which were vented at frequent intervals, gas production profiles were higher than the

corresponding profiles obtained using the Menke *et al.* (1979) technique; where fermentations were conducted in gas-tight syringes and gas production was monitored by the ascent of the syringe plunger.

Secondly, two potential applications for the technique were investigated; (1) as a routine feed analysis tool for the prediction of the digestible energy (DE) content of equine feeds and (2) as a screening method for investigating the potential use of novel feed additives. The first application was investigated by incubating sixteen feedstuffs of known DE. Gas production parameters, DM loss and VFA production were then used to derive prediction equations. The best prediction equation was DE = -0.68 + 0.01087 DML + 6.82 Z - 2.297 log L_T (R^2 = 0.878; RSD = 0.99; where DML is dry matter loss *in vitro*, Z is a rate parameter and L_T is the lag time for gas production). The second application was investigated using four antibiotics; monensin, avoparcin, penicillin G and chloramphenicol in the automated gas production system (APES; Davies *et al.*, 1995). Differences in gas production profiles were detected between antibiotic supplemented and control fermentations suggesting that the technique can be used to screen feed additives.

In vitro gas production techniques show considerable potential as routine analytical tools for animal nutrition studies. Results presented in this thesis inform the development of standardised methodologies and procedures for use during *in vitro* gas production studies, thus enabling this technique to be adopted as a robust, reliable and routine analytical tool in animal nutrition studies.

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LIST OF ABBREVIATIONS

A	asymptote of gas production
Ac	acetic acid
ABPE	acute bovine pulmonary oedema and emphysema
ADF	acid detergent fibre
AFRC	Agricultural and Food Research Council
APES	automated pressure evaluation system
ATP	adenosine triphosphate
b and c	gas production rate constants
B	gas production parameter (France *et al.*, 1993)
BP	British Pharmacopoeia
Bu	butyric acid
°C	degrees Celcius
CH_4	methane
CO_2	carbon dioxide
Co-EDTA	cobalt - ethylenediaminetetra - acetic acid
CP	crude protein
Cr-EDTA	chromium - ethylenediaminetetra - acetic acid
DE	digestible energy
d.f.	degrees of freedom
DM	dry matter
DMD	dry matter degradation
DMIV	apparent dry matter digestibility *in vivo*
DML	dry matter loss *in vitro*
DMSO	dimethyl sulphoxide
E	extent of degradation in the rumen
E	activation energy
FRGP	fractional rate of gas production at the time when 50 % of the total gas volume has been produced
g	gramme(s)

gs	gravity
GE	gross energy
GMD	geometric mean diameter
GPT	glutamate pyruvate transaminase
h	hour(s)
H_2	hydrogen
IGER	Institute of Grassland and Environmental Research
K	Kelvin temperature
k	passage rate constant
Ka	acid dissociation constant
kg	kilogramme(s)
kPa	kilopascal(s)
λ	mean free path
l	litre(s)
L	Avogadro's constant
L_T	lag time
LDH	lactate dehydrogenase
LED	light emitting diode
LSD	least significant difference
M	molar mass
m	gradient of a line
MADF	modified acid detergent fibre
mb	millibar(s)
MDS	microwave digestion system
MHz	mega Hertz
min	minute(s)
MJ	megajoule(s)
ml	millilitre(s)
MLP	maximum likelihood programme
mg	milligramme(s)
mm	millimetre(s)

mmol	millimole(s)
mol	mole(s)
MPE	mean prediction error
m.p.h	miles per hour
ms	millisecond
MSPE	mean square prediction error
MT	Menke *et al.* (1979) technique
n	amount of substance
N	nitrogen
NAD	nicotinamide adenine dinucleotide
NDF	neutral detergent fibre
NIR	near infrared reflectance spectroscopy
nm	nanometre(s)
NO	naked oats
NSP	non starch polysaccharide
O and P	means of the actual and predicted values, respectively
OF	oatfeed
OM	organic matter
OMD	organic matter digestibility
P	pressure
PC	personal computer
pKa	the negative logarithm of the acid dissociation constant
ppm	parts per million
Pr	propionic acid
psi	pounds per square inch
PTT	pressure transducer technique
Q and Z	gas production parameters (France *et al.*, 1993)
Q_{10}	temperature coefficient
r	correlation coefficient
R	molar gas constant
RG	ryegrass

RUSITEC	rumen simulation technique
s	second(s)
SB	unmolassed sugar beet pulp
s.e.	standard error
s.e.d.	standard error of difference
SH	soya hulls
SMLR	stepwise multiple linear regression
S_O^2 and S_P^2	variances of O and P respectively
s.t.p	standard temperature and pressure
T	temperature
t	time
TVFA	total volatile fatty acid
t_{50}	time taken to produce 50 % of the total gas production
t_{95}	time taken to produce 95 % of the total gas production
μ	fractional rate of gas production
μl	microlitre(s)
μmol	micromole(s)
u	root mean square speed
u^2	mean square speed
V	volume
V_m	molar volume
v/v	volume in volume
val	valeric acid
VFA	volatile fatty acid
x_A and x_B	molar fractions of gases A and B
y	cumulative gas production at time t
z	the frequency with which gas molecules collide

CHAPTER 1 - INTRODUCTION

Formulating diets for farm animals requires accurate information about both the nutrient requirements of the animal and the nutritive value of feedstuffs, in order that the animal may meet the various levels of performance and production imposed upon it. The nutrient requirement of an animal refers to the amount of that nutrient which an animal requires for a particular function, for example maintenance, growth or lactation. Nutrient requirements are usually obtained from feeding trials where different levels of nutrients are fed and the level which produces optimal performance is determined. However, factorial approaches which utilise the nutrient costs for maintenance and production or computer models which simulate the animal may also be used (Block, 1996). The nutrient requirement of an animal varies with age, sex, state of health and the level of work / production (for example, meat, milk or wool production). In addition there is individual variation between animals. Recommendations for nutrient requirements for animals in any particular state, such as growth or lactation, therefore, include a safety margin to allow for variation between individual animals. The term used to describe the nutrient requirement after a safety margin has been added is the nutrient allowance. The aim of the safety margin is to ensure that all animals receive a sufficient quantity of the nutrient (McDonald *et al.*, 1995; Oldham, 1996). In the United Kingdom detailed tables of nutrient allowances for farm animals (poultry, ruminants and pigs) have been produced by the Agricultural Research Council (1975, 1980, 1981 & 1984).

Once the nutrient requirements of an animal are known, a diet which provides the correct balance of nutrients can be formulated if accurate information on the nutritive value of feeds is available. Plants and plant products compose the bulk of farm animal diets, however products of animal origin such as fishmeal and milk have also been fed in limited amounts (McDonald *et al.*, 1995). Plants are composed of carbohydrates, proteins, lipids, nucleic acids, organic acids, vitamins and minerals. The quantity of each constituent varies according to the species of plant, type of plant material (for example, leaf or stem) and the stage of maturity. The carbohydrate

portion generally constitutes the greatest proportion of the plant and consists of sugars, starch, non-starch polysaccharides and complex carbohydrates (glycolipids and glycoproteins). There are two main functions of the carbohydrate portion; (1) structural (plant cell walls being composed mainly of cellulose) and (2) storage (energy is usually stored by the plant in the form of starch and fructans). The carbohydrate storage components of the plant are generally easily digested by the animal whilst the structural components are less readily degraded. Therefore analysis of the feed must take this into consideration. Analysis of feedstuffs usually involves determining the dry matter (DM), organic matter (OM), crude protein (CP) and fibre content of the feedstuff. DM is determined by drying a known weight of food to constant weight at 100 °C, whilst determination of OM involves heating a known weight of feedstuff at 500 °C until all the carbon has been removed. The weight of the residue (or ash) which is left is subtracted from the weight of DM which was heated to give the OM content of the feed. Crude protein is calculated using the Kjeldahl method. This involves treating the feed with sulphuric acid to convert the nitrogen present to ammonia. The ammonia is then liberated, by the addition of sodium hydroxide, and collected in standard acid. As the method measures nitrogen rather than protein, the quantity of nitrogen in the ammonia which is collected is multiplied by 6.25 (it is assumed that the nitrogen is derived from protein containing 16 % nitrogen) to obtain an approximate protein value (McDonald *et al.*, 1995). There are several methods available for determining the fibre composition of feedstuffs. The most widely used method of analysing fibre is that described by Goering and Van Soest (1970). This technique involves determining the fibre portion of the feed using neutral- or acid-detergent. The neutral-detergent procedure for cell wall constituents involves boiling the feed with neutral solutions of sodium lauryl sulphate and ethylenediaminetetraacetic acid (EDTA). The residue which remains is termed the neutral-detergent fibre (NDF) and consists mainly of lignin, cellulose and hemicellulose. The acid-detergent fibre procedure involves boiling the feed with 0.5 mol l^{-1} sulphuric acid and cetyltrimethylammonium bromide. The residue which remains is termed the acid-detergent fibre and consists of crude lignin, cellulose and silica. As these are gravimetric techniques the exact chemical composition of the

NDF and ADF residues are not known (Wolters *et al.*, 1992). The fibre portion of the feed may be more accurately described in chemical terms by non-starch polysaccharide (NSP) analysis as described by Englyst and Cummings (1984); whereby alditol acetate derivatives of carbohydrate monomers derived from acid hydrolysis of washed, polymeric, de-starched samples, are quantified by gas chromatography. In addition to obtaining details of the chemical composition of the fibre, the fibre values measured as NSP are independent of food processing and storage, and hence the amount of fibre present in processed feeds and mixed diets can be determined easily (Englyst, 1989). Near infrared reflectance spectroscopy (NIR) has also been adopted for determining the composition of feedstuffs (Norris *et al.*, 1976; Fairbrother & Brink, 1990; Schenk & Westerhaus, 1994). In terms of accuracy, precision, speed and unit cost of analysis the NIR technique, provided it is calibrated correctly, is preferable to the traditional laboratory methods mentioned above. However, the technique ultimately relies on a set of standard samples whose composition has been determined by traditional methods (Van Soest, 1982).

In addition to information on the chemical composition of the feed, it is also important to know the digestibility of the nutrients present. Several techniques have been developed to characterise feedstuffs in terms of their digestibility, for example, *in vivo*, *in situ* and *in vitro*. These techniques are discussed in chapter 2 of this thesis. *In vivo* measurements provide the standard measure of digestibility, however, for routine feed analysis *in vitro* techniques are preferable as they allow large numbers of samples to be analysed at any one time and are more rapid and less costly than *in vivo* measurements. In 1979 Menke *et al.* described an *in vitro* technique whereby the gas produced during the incubation of a feedstuff with buffered rumen fluid was used to predict the digestibility of that feed. As well as allowing the extent of digestion to be predicted the technique also provided a simple method of evaluating the rate of digestion. Hence, gas production has become a popular tool for investigating the rate and extent of digestion of ruminant feeds. This interest has led to several innovations in equipment design which have produced accurate, reliable methods of measuring gas production. Although gas production can be measured accurately, information on

where the gas comes from and the factors which influence the volume of gas recorded are still limited. Thus the interpretation of gas production data is often inaccurate.

The scope of this thesis was to investigate some of the physical, biological and chemical factors which affect the measurement of gas in gas production studies, particularly when using the manual pressure transducer technique of Theodorou *et al.* (1994). The research was undertaken with regards to suggesting a standard protocol and potential applications for the technique.

In the review of the literature, gas production techniques and gas theory in relation to the nature of the gaseous state were described in detail. In respect of this information the experimental considerations were as follows;

1. Firstly, the physical factors; temperature, head-space pressure and shaking movement, and their effect on gas production were investigated (chapter 4). These experiments were carried out using the manual pressure transducer technique. The effect of temperature was investigated by incubating four identical series of bottles, containing perennial ryegrass hay (*Lolium perenne*), at either 25, 30, 39 or 45 °C. The effect of head-space pressure was investigated using a series of experiments whereby gas production was recorded every 2, 4 or 6 h during the incubation of naked oats (*Avena nuda*) or perennial ryegrass hay (*Lolium perenne*). The effect of shaking movement was determined using three identical series of bottles; bottles in series 1 were shaken continuously, those in series 2 were shaken intermittently whilst those in series 3 were not shaken. In each section the resulting gas production profiles, DM loss and volatile fatty acid (VFA) production from each treatment were compared.

2. Secondly, biological and chemical factors which affect gas production, including the nature of the feedstuff, the culture medium and inocula source were investigated (chapter 5). Again the manual pressure transducer technique was used to investigate

these factors. VFA production along with measurements of acidification gas production were used to predict gas production. Gas production from the soluble fraction of both naked oats (*Avena nuda*) and perennial ryegrass hay (*Lolium perenne*) was also investigated. The resulting gas production profiles were compared with those from incubation of complete (soluble + insoluble) substrate.

3. Thirdly, the effect of different sources of microbial inoculum on the gas production profiles obtained during incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp were investigated. Three series of identical bottles were used and inoculated with microbial inoculum prepared from either bovine rumen fluid, equine caecal fluid or equine faeces. The resulting gas production profiles and associated measurements for each feedstuff were then compared between the different inoculum sources (chapter 5).

4. The effect of processing the test sample, in terms of particle size and method of drying, were also investigated (chapter 6). The effect of particle size was investigated using both naked oats and hay. The naked oats were divided into the following particle sizes; whole, half, quarter, coarsely ground and finely ground, whilst the hay sample was ground through a 4 mm mesh then sieved through a cascade of sieves with the following mesh sizes; 2.4, 1.2, 0.6, 0.3 and 0.15 mm. The effect of drying was investigated using a fresh sample of perennial ryegrass (*Lolium perenne*) and samples which had been microwave, freeze or oven dried. Again the resulting gas production profiles, DM loss and VFA production were compared between the different treatments.

5. The influence of the technique used to measure gas production was examined by comparing the gas production profiles from three feedstuffs (naked oats, oatfeed and ryegrass) derived using a standard protocol with the pressure transducer technique (Theodorou *et al.*, 1994) and Menke *et al.* (1979) technique (chapter 7).

6. Finally, two applications for gas production techniques were investigated (chapter 8);

a. the ability of the technique to predict *in vivo* dry matter digestibility (DMIV) and digestible energy (DE) values for equine feeds. This was investigated using sixteen feedstuffs with known DMIV and DE contents. Prediction equations containing cumulative gas volumes, fitted and derived gas production parameters, VFA parameters and DM loss were developed to predict DMIV and DE.

b. the use of the automated pressure evaluation system (APES; Davies *et al.*, 1995) for investigating the effect of antibiotics on rumen fermentation. The antibiotics studied were avoparcin, penicillin G, monensin and chloramphenicol.

CHAPTER 2 - LITERATURE REVIEW

2.1 Techniques for estimating the digestibility of animal feeds.

2.1.1 Introduction

Feed evaluation is an important part of formulating diets for domestic animals, in order that they can meet the varying requirements imposed upon them, such as reproduction and meat, milk or fibre production. Chemical analysis can be used to determine the composition and potential nutritive value of a feed. However the quantity of each nutrient which can be digested by the animal will be affected by several factors, such as the physical structure of the feedstuff, the structure and form of the nutrients in the feedstuff and the association of the nutrient with anti-nutritive factors, such as polyphenolic tannins and/or lignin (Russell & Hespell, 1981; Chesson, 1988 & 1993; Wilson, 1990; McAllister *et al.*, 1994; McDonald *et al.*, 1995; Chesson & Forsberg, 1997). In addition, the feed will be modified by digestion as it passes through the digestive tract and losses associated with the metabolic processes involved will occur. For example, the production of methane and heat of fermentation (Hungate, 1966). Due to the economic importance of feeding farm animals, several different techniques for estimating the digestibility of animal feeds have been developed. These techniques are chemical and / or biological in origin and include *in vivo*, *in situ* and *in vitro* procedures; all have their advantages as well as their disadvantages. This review discusses the most commonly used techniques for estimating digestibility, and describes in detail the *in vitro* gas production method of Theodorou *et al.* (1994) which formed the basis for the experiments reported in this thesis.

2.1.2 *In vivo* techniques for measuring digestibility

The most accurate method for determining the digestibility of a feed ('that proportion of a food which is not excreted in the faeces and which is, therefore, assumed to be absorbed by the animal' [McDonald *et al.*, 1995]) is to feed it to the animal - *in vivo*. *In vivo* procedures have been extensively reviewed by Schneider and Flatt (1975) and Cochran and Galyean (1994). The apparent digestibility of a feed can be determined

by measuring the quantity of feed consumed and the quantity of faeces produced (Cochran & Galyean, 1994; McDonald *et al.*, 1995). The proportion of the feed which is not excreted is assumed to be absorbed by the animal and is termed the apparent digestibility of the feed.

The apparent digestibility is not the true digestibility of the feed since in addition to undigested feed constituents, the faeces also contains undegraded microbial biomass and a non-dietary, or endogenous fraction. This is largely composed of substances that are secreted into the gut and not reabsorbed, together with cellular material abraded from the lining of the gut epithelium (McDonald *et al.*, 1995). The true digestibility can be calculated by subtracting the microbial and endogenous fractions from the total amount of faeces to determine the actual amount of undigestible dietary residue. However, the microbial and endogenous content of the faeces is difficult to measure and therefore determination of apparent digestibility is considered satisfactory (McDonald *et al.*, 1995).

When conditions preclude the measurement of feed intake or total faecal output from an individual animal, for example when animals are group-fed or are in a grazing situation, dietary markers/indicators can be employed (McDonald *et al.*, 1995). The use of markers has been reviewed by Faichney (1975), Kotb and Luckey (1972), Warner (1981), Fahey and Jung (1983), Dove and Mayes (1991) and Owens and Hanson (1992). There are several criteria which must be met before a substance can be used as a dietary marker; (1) the marker must not be absorbed (i.e. it must be completely indigestible), (2) it must not be affected by, nor affect, the chemical, physical or metabolic conditions of the digestive tract and its microbial population, (3) it must be either physically similar to the feedstuff under investigation or intimately associated with it and (4) it must have a specific method of analysis that does not interfere with other desired analyses (Crooker *et al.*, 1982). Dietary markers can be natural constituents of the feed (internal markers) such as lignin (Fahey & Jung, 1983), neutral detergent fibre or acid detergent fibre (Cochran *et al.*, 1986) or alkanes (Dove & Mayes, 1991; Oharjuruka & Palmquist, 1991). Alternatively, they

can be chemicals (external markers) which are mixed with the feed such as chromium (Uden *et al.*, 1980), or the rare earth chemicals like lanthanum, samarium and ytterbium (Crooker *et al.*, 1982; Teeter *et al.*, 1984). The most commonly used external marker is chromium in the form of chromic oxide Cr_2O_3, which is highly insoluble and is unlikely to be present as a natural constituent of feeds (McDonald *et al.*, 1995). The marker is mixed with the feed at a known concentration and fed continuously or frequently (at least once daily) to the animals throughout the study. When the marker has reached equilibrium in the faeces, usually after 5 to 7 days (Brandyberry *et al.*, 1991; Owens & Hanson, 1992), faecal samples are collected and the concentration of the marker in the faeces is measured. Faecal sampling usually involves taking two daily samples over a collection period of 7 days (Cochran & Galyean, 1994); the total collection of faeces is not required. The apparent digestibility of DM is determined by subtracting the concentration present in the feed from the concentration in the faeces and dividing by the concentration in the faeces (equation 2.1; McDonald *et al.*, 1995).

Apparent DM digestibility =

(g marker kg^{-1} faeces - g marker kg^{-1} food) / g marker kg^{-1} faeces

(equation 2.1)

Markers can also be used to determine the rate of passage of a feedstuff and the flow rate of digesta through the digestive tract (Cochran & Galyean, 1994). The rate of passage refers to the speed with which the feedstuff passes through the digestive tract whilst the flow rate refers to the volume of digesta which passes from one section of the digestive tract to another in a given time. The rate of passage of the feed through the digestive tract can be determined from the observed change in marker concentration with time in the faeces (Grovum & Williams, 1973).The marker is fed as a pulse dose and its concentration in the faeces is measured at regular time intervals post dosing. Faecal collection periods post dosing usually last approximately 7 days in ruminants with faecal samples taken every 2 - 3 h initially

(due to an adaptation period of two weeks, followed by a collection period of 7 - 10 days; Cochran & Galyean, 1994).

2.1.3 The *in situ* bag technique for estimating digestibility

The *in situ* bag technique (also known as the *in sacco*, artificial fibre / nylon / Dacron bag technique) was developed by Quin *et al.* in 1938 and has become a popular tool for evaluating the rate and extent of degradation of feeds in the rumen. The technique involves the incubation of a feedstuff, contained in a material bag (originally silk but quickly replaced with artificial fibres such as nylon or Dacron), in the rumen of an animal. The disappearance of feed constituents, such as dry matter (DM), organic matter (OM), crude protein (CP), nitrogen (N) and fibre (ADF or NDF), from the bag is measured following incubation in the rumen for varying periods of time. The time course of degradability for a sample can then be described using the mathematical modelling procedures adopted by Ørskov and McDonald (1979) (see also section 2.3 for further details of the model).

The *in situ* bag technique requires the use of fistulated animals, in order that the bags and their contents can be incubated in the rumen. Rumen fistulation involves creating an opening between the posterior dorsal sac of the rumen and the animal's flank. Techniques for fistulating the rumen were first described by Fluorens in 1833 (Hungate, 1966), since this time several fistulation procedures have been developed. The fistulation procedures of Schalk and Amadon (1928) and Jarrett (1948) have been described in detail by Johnson (1966). Modern fistulas are generally fitted with a cannula (a permanent plastic or rubber tube with a screw cap) and thus the rumen is kept closed to the external environment for the majority of the time. The first cannula (or 'closed fistula') for use in ruminants was described by Quin *et al.* (1938), before this time fistulas were generally left open to the outside environment.

There are several factors which influence the degradability of a feed sample *in situ*. These include (1) the physical characteristics of the bag, such as the type of material and its pore size (Weakley *et al.*, 1983; Uden & Van Soest, 1984), (2) the physical

(or when the animal defecates) becoming less frequent towards the end of the faecal collection period (Kotb & Luckey, 1972).

The digesta present in the digestive tract of ruminants is composed of both a particulate and a fluid - phase. Therefore, when estimating the flow rate of digesta through a particular stage of the digestive tract, for example, the duodenum, or the passage rate of a feed which is composed of both a liquid and a solid component a dual phase marker system may be the most accurate method (Titgemeyer, 1997). Dual phase markers involve the use of a fluid - phase and a particulate - phase marker simultaneously (Faichney, 1975). The digesta is sampled at certain time intervals after both the fluid and particulate - phase markers have been administered. The marker concentration in each phase of the digesta sample is then measured and combined with the other to give a flow rate for the 'whole' digesta sample or used to determine the passage rate of that feed constituent (Faichney, 1975). Chromium - ethylenediaminetetra - acetic acid (Cr-EDTA) and cobalt - ethylenediaminetetra - acetic acid (Co-EDTA) are the most common markers for estimating both digesta flow rate and digesta passage rate in the fluid phase (Faichney, 1986; Huhtanen & Kukkonen, 1995; Perez *et al.*, 1995; Amici *et al.*, 1997). Chromium and the rare earth elements (particularly ytterbium) are often used to estimate the digesta flow rate and digesta passage rate of the particulate phase (Crooker *et al.*, 1982; Stefanon *et al.*, 1992; Huhtanen & Kukkonen, 1995; Perez *et al.*, 1995; Titgemeyer, 1997). Spores from *Bacillus stearothermophilus* have also proven to be useful markers for estimating the passage rate of the particulate phase (Mir *et al.*, 1997). Whilst Egan and Doyle (1984), concluded that lignin was preferable to ^{103}Ru -labelled tris - (1, 10 - phenanthroline) - ruthenium II chloride (^{103}Ru-P) as a particulate - phase marker when sheep were fed certain roughages.

Although, *in vivo* procedures produce accurate estimates of feed digestibility, they have several disadvantages. They are labour intensive, costly in terms of feed and animals and results are not obtained until four weeks after the start of the experiment

nature of the sample, for example, dried versus fresh and/or particle size (Weakley *et al.*, 1983; Emanuele & Staples, 1988; Lopez *et al.*, 1995), (3) sample size to bag surface ratio (Mehrez & Orskov, 1977; Lindberg, 1981; Uden & Van Soest, 1984; Uden, 1992), (4) the diet of the host animal (Nocek, 1988; Weiss, 1994) and (5) the washing and drying procedure for the bags following incubation (Cherney *et al.*, 1990; Hyslop, 1991). The influence of these factors on the *in situ* procedure have been extensively reviewed by Nocek (1988), Michalet-Doreau and Ould-Bah (1992), Weiss (1994) and Huntington and Givens (1995).

The general consensus from these reviews has been to emphasise the importance of standardising all of the above factors when conducting *in situ* studies. However, the ultimate recommendations are dependent upon the aim of the experiment. One specialised application for the *in situ* technique is in the assessment of protein degradation in the rumen, having been adopted by the AFRC (1992) as the standard method of characterising rumen degradability of nitrogen. Hence the AFRC (1992) have detailed recommendations on the procedures which should be followed when conducting *in situ* studies for this purpose. These recommendations include the physical characteristics of the bag, such as size, type of material and pore size, substrate preparation (the method which should be adopted to dry and particle size the sample), the diet of the host animal and the washing procedure for the bags following incubation.

2.1.4 *In vitro* techniques for estimating digestibility

In vitro techniques for routine feed analysis must be rapid, simple to perform, reproducible and produce reliable results. These techniques are used to predict *in vivo* digestibility and are desirable in terms of cost and the number of samples which can be analysed at any one time. They rely upon simulating the rumen environment. In the case of the *in situ* technique, the test sample will be suspended in 'real' rumen environmental conditions in terms of pH, temperature, buffer substrate, enzymes and microbial environment. *In vitro*, all of these conditions must be simulated (Nocek, 1988).

In order to predict the *in vivo* digestibility of animal feeds from *in vitro* procedures calibration sets are required. A calibration set consists of several feedstuffs which have known *in vivo* digestibility. The *in vitro* digestibility of these feeds is then determined and a prediction equation is developed by regressing the *in vivo* data against the *in vitro* data for the samples (Goldman *et al.*, 1987). The resulting equation can then be applied to predict the *in vivo* digestibility of other samples which have been analysed using the same *in vitro* technique. The accuracy of the prediction can be increased by developing separate equations for different forage species. For example, Tilley and Terry (1963) found that the standard error of the *in vivo* digestibility prediction was smaller when separate equations were used for legumes and grasses, compared to a single equation. Omed *et al.* (1989) also recommended that separate equations should be used for different classes of forage. Experimental variation has also been reported between laboratories using the same technique (Johnson, 1966; Weiss, 1994; Huntington, 1995), therefore ideally prediction equations should be devised by each individual laboratory for the feeds tested in that laboratory. In addition, if any of the processes involved in the technique change new equations should be developed in order to account for these changes (Weiss, 1994).

The use of standards is also an essential part of the *in vitro* technique. Standard samples should be included in each experimental 'run', in order to ensure that the results obtained from each run are representative of the technique. Ayres (1991) stated that standard samples should be used to correct between run variation by, firstly, determining the normal amount of between run variation. Once the normal variation (95 % confidence interval) has been determined, any results which are outside the normal between run variation can be corrected (using the standards as a reference). However, if the standards deviate greatly (from either the normal variation or from each other) the results should be discarded and the *in vitro* analysis repeated.

2.1.4.1 Incubation with rumen fluid

Incubation with rumen fluid is one of the most commonly used methods for estimating *in vivo* digestibility in the laboratory (*in vitro*). A microbial inoculum prepared from rumen fluid ensures that the micro-organisms used to degrade the feed sample will be representative of those in the rumen. The use of rumen fluid necessitates the use of fistulated animals, however, alternative sources of inoculum, such as the microbial populations in faeces, can be used (El Shaer *et al.*, 1987; Akhter *et al.*, 1994 & 1995; Harris *et al.*, 1995).

In general, digestibility determined *in vitro* is slightly lower than that determined *in vivo*, largely due to the *in vitro* techniques being unable to properly simulate the animals complex digestive system. Hence, corrective regression equations have been devised to relate *in vitro* digestibility to *in vivo* digestibility (Weiss, 1994; McDonald *et al.*, 1995).

In vitro techniques which rely on the use of rumen fluid can be divided into two types employing either batch or continuous culture systems.

2.1.4.1.1 Batch culture

The most commonly used *in vitro* method for predicting the *in vivo* digestibility of a feedstuff for ruminants is that of Tilley and Terry (1963). This is a two-stage procedure which involves incubating a small, finely ground sample of the test feed (ca. 0.5 g) with 10 ml of rumen fluid and 40 ml of a bicarbonate buffered, salt solution, under anaerobic conditions for 48 h (anaerobic techniques for the cultivation of anaerobic rumen bacteria are detailed in section 3.5). The sample is then acidified with hydrochloric acid (to pH 2) and incubated with pepsin for a further 48 h to simulate postruminal digestion. The incubation buffer, like most buffers used in rumen *in vitro* digestion procedures is based upon the composition of ruminant saliva as described by McDougall, (1949).

2.1.4.1.2 Continuous culture

Unlike batch cultures, which are closed systems where fermentation end-products are allowed to accumulate with time and are not removed during incubations, continuous culture systems are open and provide a continuous supply of fresh buffer solution (and feed) to the culture as spent residues and micro-organisms are removed from the culture and this helps prevent the build up of toxic end-products. Thus, the continuous cultures more closely resemble ruminal conditions, enabling the maintenance of rumen protozoal populations at levels comparable to *in vivo* systems; protozoa are often absent from batch cultures because rumen conditions are not properly simulated (Stewart *et al.*, 1961; Weller & Pilgrim, 1974).

The most frequently used continuous culture system in ruminant nutrition is RUSITEC (RUmen SImulation TECnique) developed by Czerkawski and Breckenridge (1977). Once the cultures are stable (5 - 7 days after inoculation), they can be maintained indefinitely, allowing the effect of factors such as dilution rate, increased feeding level and dietary changes to be investigated (Czerkawski & Breckenridge, 1977).

RUSITEC and other continuous-flow systems are technically complex in comparison to closed batch cultures and usually involve long-term studies with only a few vessels. They are therefore perhaps better suited to investigating factors which affect the processes of digestion as opposed to being used as a routine procedure for estimating *in vivo* digestibility.

2.1.4.2 Enzymatic methods

In vitro digestibility techniques, based on enzymes, have been developed in order to try to reduce the variability between predicted values of *in vivo* digestibility caused by variation in rumen fluid samples (due to host diet and animal to animal variation).

Aerobic fungi, such as *Trichoderma* and *Aspergillus* spp. produce a wide range of hydrolytic enzymes that can digest the major structural carbohydrates of plant cell-

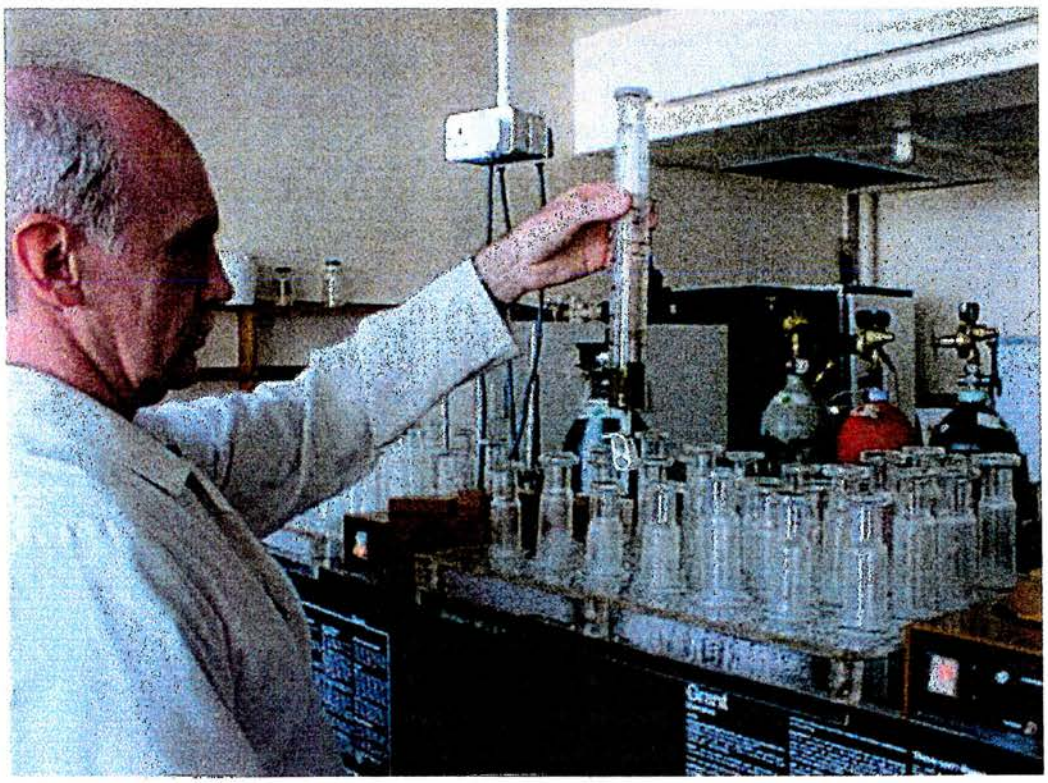
walls. The enzymes used in enzymatic digestibility techniques are generally commercially available fungal cellulases and hemicellulases (McQueen & Van Soest, 1975; Minson, 1990; Weiss, 1994). The enzymes are used in a similar way to rumen fluid; a small, finely ground sample of feedstuff being incubated in a buffer solution containing the enzyme. The technique has developed in a similar way to the Tilley and Terry (1963) technique, in that incubation with pepsin - HCl is required, in addition to incubation with the cellulolytic enzyme, to increase the accuracy of the digestibility predictions (Jones & Hayward, 1975). However, unlike the Tilley and Terry (1963) technique, where the pepsin - HCl incubation follows the incubation with rumen fluid, in the method of Jones and Hayward (1975) the pepsin - HCl treatment precedes the enzymatic step.

The accuracy of *in vitro* enzymatic techniques appears to depend upon both the nature of the feedstuff in question and the enzyme(s) used in the incubation. Generally, they are less accurate than those using rumen fluid. Enzymatic digestion techniques may, therefore be more suitable for measuring relative differences in digestibility between feedstuffs rather than absolute digestibility values (Nocek, 1988; Stern *et al.*, 1997). As they do not utilise rumen fluid they may also be useful were access to fistulated animals is not possible.

2.1.4.3 The gas production technique for estimating feed digestibility

During the fermentation of feedstuffs by rumen micro-organisms, volatile fatty acids (VFA) and gas are produced. Menke *et al.* (1979) were the first to use gas production during the fermentation of a feedstuff to estimate *in vivo* apparent digestibility. The apparatus used to measure gas production is shown in Plate 1. In their procedure, fermentations are conducted in 100 ml ground-glass syringe barrels containing a weighed quantity of the feedstuff (200 - 300 mg) in 20 ml of buffered, anaerobic medium and 10 ml of rumen fluid. The production of gas during the fermentation causes the syringe plunger to rise inside the barrel and the total volume of gas

Plate 1 The Menke *et al.* (1979) technique for measuring gas production.



Approximately 300 mg of feedstuff are fermented in 100 ml ground-glass syringe barrels, which are situated in a waterbath at 39 °C. The production of gas during the fermentation causes the syringe plunger to rise inside the barrel and the volume of gas produced can be recorded from the graduations on the syringe barrel.

produced can be correlated with the degradation of the feedstuff. Unlike, other *in vitro* techniques, where the end-point of fermentation is measured directly using gravimetric techniques, gas production is an indirect measure of dry matter degradation (DMD). In addition to the end-point of fermentation, the rate of fermentation can also be determined using gas production techniques, simply by measuring the rate of gas evolution. In the Menke *et al.* (1979) procedure the rate of gas production is measured by recording the position of the syringe plunger at defined intervals throughout the incubation. At the end of the fermentation dry matter (DM) disappearance can be determined by filtering the syringe contents, then drying and reweighing the syringe contents. Other *in vitro* techniques, such as the Tilley and Terry (1963) technique, may also be used to determine the rate of fermentation. This can be done by arresting the fermentation at pre-determined times and measuring the loss of DM using gravimetric procedures. However, this approach requires destructive sampling of harvested cultures and is more labour intensive than gas production procedures which are non-destructive and follow the same sample (and replicates) throughout the entire incubation. The end-point techniques may also be less accurate than gas production techniques for determining the rate of fermentation, as they rely upon DM loss measurements. Ruminant feeds can contain significant quantities of soluble, but undegradble components which will be considered as fermented in DM loss digestibility procedures, but in gas production techniques, however, any material which has not been fermented will not contribute to the gas pool.

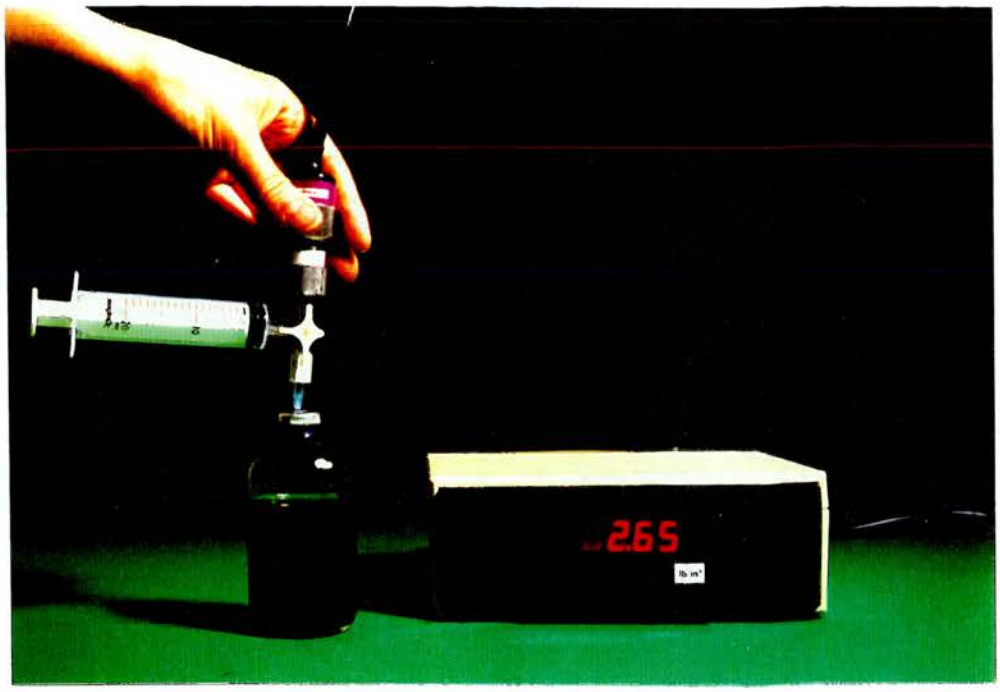
Feed intake is an important factor in animal production; the more an animal eats the greater the opportunity to increase production (McDonald *et al.*, 1995). The rate of fermentation is one of several factors which affects feed intake; the faster a feed can be fermented and be absorbed as end products through the digestive tract, the greater the opportunity for increased feed intake. Working with several cereal straws, Blummel and Orskov (1993) found that the rate of fermentation was highly correlated to feed intake. Hence, measuring the rate of fermentation of a feed, via gas production, may be useful in predicting animal performance on that feed.

Since the development of the Menke technique (1979), several other procedures have been developed in order to study the kinetics of rumen fermentation and to predict digestibility. These newer techniques all use electronic devices to measure gas production, some are manual whilst others are automated, for example see Beuvink *et al.* (1992), Pell and Schofield (1993), Theodorou *et al.* (1994), Davies *et al.* (1995) and Cone *et al.* (1996).

2.1.4.3.1. Electronic measurement of gas production

There are two commonly employed manual techniques for recording gas production; the manometric Menke technique (1979) (as described above) and the pressure transducer technique (Theodorou *et al.*, 1994). The first electronic measurement of gas production for the purpose of estimating nutritional parameters of ruminant feeds was made at the Institute of Grassland and Environmental Research in 1989 - 90 (M.K. Theodorou - personal communication). The pressure transducer technique is more complex than the Menke technique. It involves incubating a small (0.2 - 1.0 g) sample of feedstuff in 160 ml gas-tight serum bottles containing 89 ml of anaerobic medium and 10 ml of rumen fluid. The bottles are sealed with butyl rubber stoppers and incubated at 39 °C. The gas produced during the fermentation accumulates in the head-space of the serum bottle as the fermentation proceeds. The pressure transducer assembly (consisting of a pressure transducer connected to a digital readout voltmeter, gas-tight syringe and needle) is then used to measure and release the accumulated gas from the serum bottles (Plate 2). This involves inserting the needle (connected to the pressure transducer assembly) through the butyl rubber stopper, into the head-space of the serum bottle. The pressure in the head-space is displayed on the light emitting diode (LED) of the voltmeter. The pressure is noted and the syringe plunger is withdrawn until the pressure reading returns to ambient pressure (0.00 ± 0.02 psi). The whole pressure transducer assembly is then removed from the head-space and the volume of gas in the syringe is noted, before being discarded into a fume cupboard. This measurement is repeated at regular intervals throughout the incubation and gas accumulation profiles are constructed by summation of the regression-corrected gas volumes.

Plate 2 The pressure transducer technique (Theodorou *et al.*, 1994) for measuring gas production



The pressure transducer assembly consists of the pressure transducer, connected via a three way valve to a syringe and a syringe needle. A voltmeter with light emitting diode (LED) displays the pressure. Gas production is measured by inserting the syringe needle through the butyl rubber stopper of a bottle. The pressure is noted from the digital display and the syringe plunger is withdrawn until the pressure in the head-space returns to ambient pressure (as indicated by a reading of zero on the digital display unit). The pressure transducer assembly is then withdrawn from the bottle, and the volume of gas in the syringe noted before being discarded into a fume cupboard.

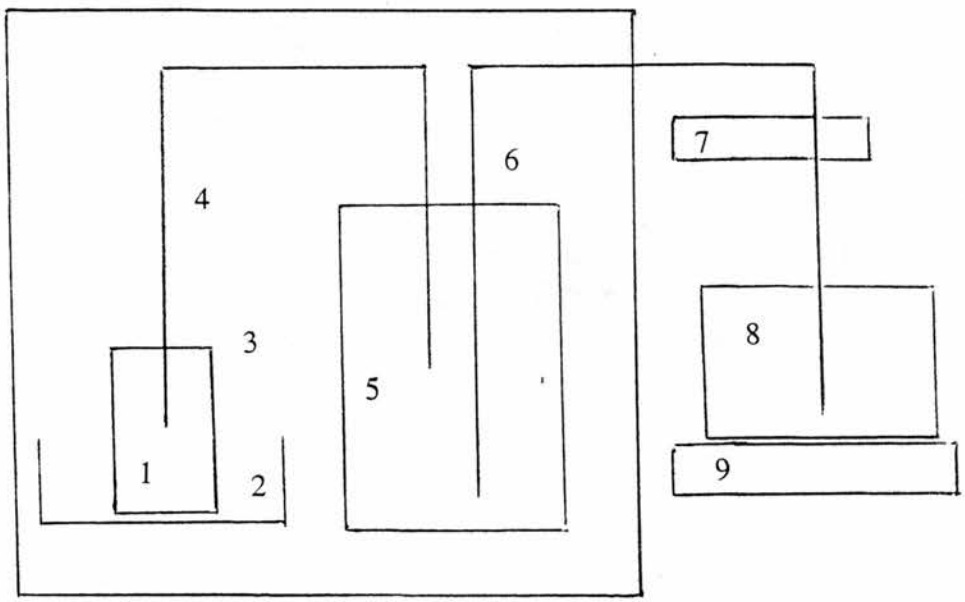
This technique gives accurate, precise results which are simple to obtain. Its main advantages over the Menke technique include more accurate measurement of small gas volumes, i.e. the Menke technique employs 100 ml syringes whereas various sizes of syringes are employed in the pressure transducer technique, and the ability to correct all gas volume measurements according to the corresponding pressure in the bottle head space. The major disadvantage of the pressure transducer technique is the high requirement for labour to record gas production at frequent intervals, particularly during the initial incubation period (this is also a problem with the Menke technique). In order to overcome this problem, several automated systems for recording gas production have been developed.

2.1.4.3.2. Automated measurement of gas production

Automation of the recording system using pressure transducers, pressure sensitive switches, solenoid valves and computer interfaces (Beuvink *et al.*, 1992; Pell & Schofield, 1993; Davies *et al.*, 1995; Cone *et al.*, 1996) has greatly increased the use of gas production systems as routine laboratory procedures to estimate both the apparent digestibility of feedstuffs and the rate of degradation.

The first automated device was developed by Beuvink *et al.* (1992) and involved a liquid displacement system (Figure 2.1.1). This system used a 100 ml serum bottle (fermentation vessel) connected to a water displacement bottle (1 litre) via butyl rubber tubing, which in turn was connected to a collection vessel placed on a balance. Gas produced in the serum bottle passed into the displacement bottle (containing 700 ml of saturated NaCl solution at pH 1) forcing liquid to stream towards the collection vessel until an equilibrium was established. The whole system involved 24 serum bottles, each with their own displacement bottle, connected through a 24 - way valve to their own overflow tube. The weight of liquid displaced by the gas was stored in a data logger. The serum bottles were held in a shaking water bath (39 °C; 50 revolutions per min) throughout the incubation.

Figure 2.1.1 Diagram of the automated gas production technique described by Beuvink *et al.* (1992)



100 ml serum bottles (1) are incubated in a shaking water bath (50 rpm; 39 °C) (2). A syringe needle (3), inserted through the butyl rubber cap of the serum bottle, is attached with butyl rubber tubing (4) to a 1 litre water displacement bottle (5) containing 700 ml saturated NaCl solution at pH 1. Tygon tubing (6) leaves the displacement bottle and passes through a 24 way valve (7) to a collection vessel (8). The collection vessel is situated on a balance (9) and the weight of liquid displaced by the gas is stored on a data logger. (Adapted from Beuvink *et al.*, 1992).

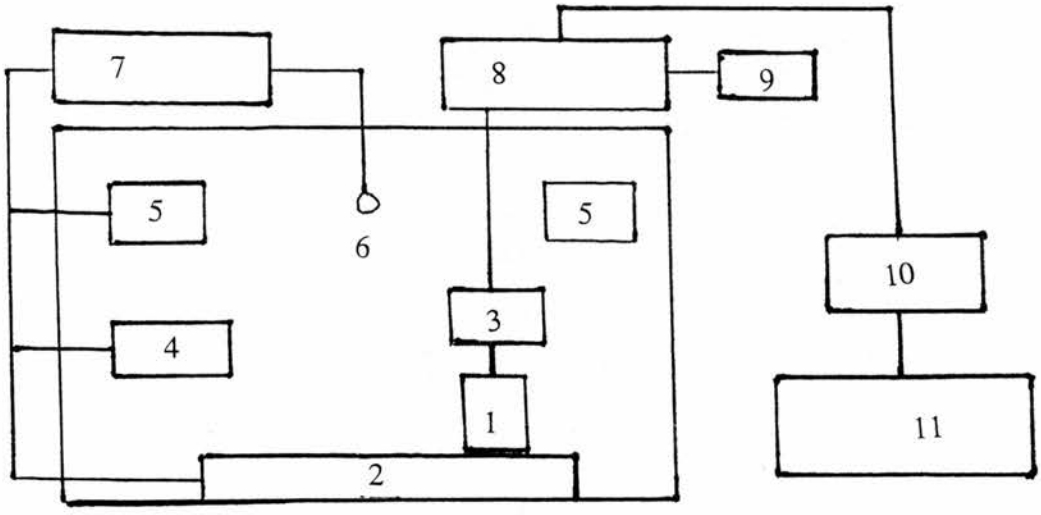
The system developed by Pell and Schofield (1993) utilised individual pressure sensors which transmit data to an IBM - compatible computer via an analogue to digital (A/D) card. The sensors are connected to the head-space of 50 ml serum bottles, which are placed in an incubator at 39°C (Figure 2.1.2). The pressure range of the sensors was from 0 to 103.4 kPa (0 to 15 psi). In this system, the bottles were allowed to equilibrate for 15 minutes, then zeroed by puncturing the stopper with a needle for 5 s. After zeroing the pressure sensors are plugged into the computer leads and the readings initiated. An interval of 1 h was allowed between readings, each reading being composed of the average of the sensor voltage measured over a 20 s interval for each bottle. The data for each bottle were displayed on the monitor and automatically written to the computer's hard disk. The sensor output was calibrated by injecting 3 ml aliquots of CO_2 gas into a bottle (x 4 replicates) and recording the pressure after each addition. A similar procedure was then followed using air instead of the highly soluble CO_2 as the added gas to generate a response curve for a gas with low water solubility (i.e. CH_4; see section 2.2.7.3.1). This data is then used to calculate a conversion factor which is used to convert the voltage readings into their corresponding gas volumes. The conversion factor is calculated using the following equation:

$$C = C_i \times C_s / (f \times C_i + (1 - f) \times C_s) \qquad \text{(equation 2.2)}$$

where C_i and C_s are the conversion factors for air and CO_2, and f is the fraction of CO_2 in the head-space gas (generally between 0.6 and 0.9).

The automated pressure evaluation system (APES) reported by Davies *et al.* (1995) differs from that of Pell and Schofield (1993) in that the bottles are vented, thereby preventing the accumulation of gas in the head-space. The system consists of 10 - 12 racks of 4 x 150 ml Duran bottles situated in a constant temperature room at 39°C (Plate 3). The bottles are connected to pressure sensitive switches which, when activated by a pressure of 4.5 kPa (0.65 psi), vent, via a solenoid valve, for three seconds to release the accumulated gas, returning the pressure in the head-space to

Figure 2.1.2 Diagram of the automated gas production technique described by Pell and Schofield (1993)



Gas production is measured in 50 ml serum bottles (1) which are incubated at 39 °C, and shaken at 48 rpm (2). The incubator consists of a heater (4), two fans (5) and a temperature sensor (6) which is connected to a temperature controller (7). The production of gas in the bottles is detected, as a change in pressure, by a pressure sensor (3). The pressure sensor is connected to an analog to digital card (10), via an interface connector (8) which is powered with 10 volts (9). The data is stored on a personal computer (11). Although only one pressure sensor is shown in the diagram, up to 15 can be connected allowing gas production to be recorded in 15 serum bottles. (Adapted from Pell and Schofield, 1993).

Plate 3 The automated pressure evaluation system (APES; Davies *et al.*, 1995) for measuring gas production.

The APES consists of 10 - 12 racks of 4 x 150 ml Duran bottles, situated in a constant temperature room (39 °C). Each bottle is connected to a pressure sensitive switch, which when activated by a pressure of 4.5 kPa vents via a solenoid valve for three seconds, releasing the accumulated gas. The time each bottle vents is recorded throughout the incubation on a personal computer.

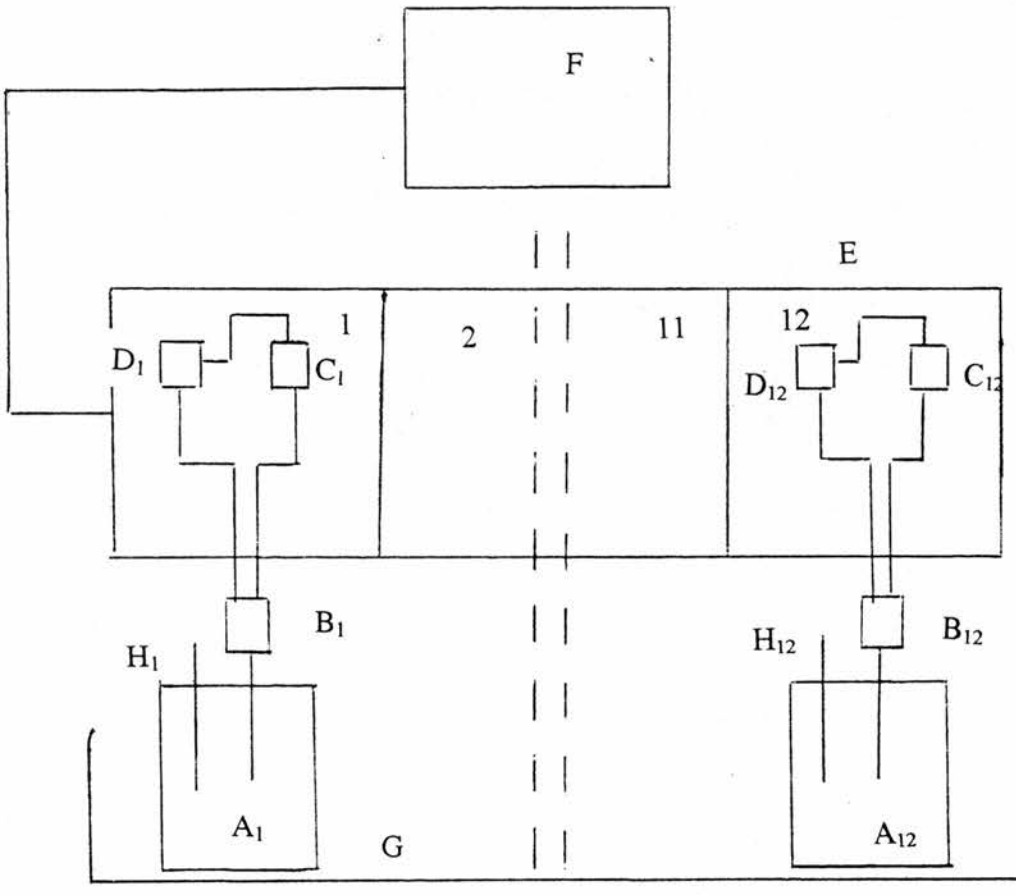
ambient. The valves are linked to an IBM compatible personal computer which automatically records the venting sequences. At the end of every gas run, each bottle is calibrated by injecting air into the bottle until the vent opens. The volume of gas which has been injected into the bottle is noted and the average of three calibrations per bottle is used to calculate the total volume of gas produced, i.e. volume of gas required to vent bottle x no. of vents for that bottle = total gas production.

The automated, time-related gas production apparatus developed by Cone *et al.* (1996) is similar to APES (Davies *et al.*, 1995). However, instead of pressure sensitive switches, pressure transducers which have a range of 0 to 2.5 kPa (measured relative to atmospheric pressure) are used. Like APES, when a certain amount of gas has been produced and detected as an increase in pressure, to a pre-determined level, the electric valves are opened and the accumulated gas is released. The pressure of gas necessary to open the valve is set to 0.65 kPa; compared to the 4.5 kPa required to open the valves in the APES system. The valves are opened for a fraction of a second (approximately 50 ms) and release approximately 0.7 ml of gas each time they open (Cone *et al.*, 1996). Opening and closing of the valves is regulated by an electronic control unit and data are transferred and stored on the hard drive of an IBM compatible personal computer.

The system consists of up to ten control units each containing 12 x 250 ml serum bottles, situated in a water bath at 39 °C (Figure 2.1.3). Each bottle was calibrated by inserting a known amount of air into the bottles and counting the number of valve openings, this was repeated ten times. Unlike the other automated systems (Davies *et al.*, 1995; Pell & Schofield, 1993), the bottles were not calibrated after every gas production experiment but instead at two weekly intervals.

The automated systems of Pell & Schofield (1993) and Cone *et al.* (1996) both involve shaking the bottles, using either intermittent stirring with magnetic bars (48 rpm) or a shaking water bath (50 rpm), respectively. According to Pell and Schofield (1993) the purpose of agitation was to avoid supersaturation of solutions with CO_2.

Figure 2.1.3 Diagram of the automated gas production system described by Cone *et al.* (1996)



Fermentations are conducted in 250 ml serum bottles (A), situated in a shaking water bath (G) (50 rpm; 39 °C). Gas produced in the bottles flows through a 50 ml glass expansion vessel (B) and is measured by a pressure transducer (C). When the pressure reaches 0.65 kPa the pressure is released by an electric valve (D). Opening and closing of the valve is controlled by an electronic control unit (E) which sends the data to a personal computer (F). Each control unit can measure gas production from twelve bottles and up to ten control units may be connected in series. H is a syringe needle which is used to equilibrate the system for 2 min at the start of the incubation. (Adapted from Cone *et al.*, 1996).

In the APES system however, the bottles are not shaken because they are continually vented and therefore supersaturation of solutions should not occur (Morris, 1983).

The use of a venting mechanism, in both the Davies *et al.* (1995) and Cone *et al.* (1996) systems releases the accumulated gas and hence removes the problem of end-product inhibition in terms of gas production. As Pell and Schofield (1993) do not vent their bottles, strict guidelines are required regarding substrate quantity and buffer volumes to avoid problems of gas accumulation in the head-space and exhaustion of the buffer.

Choosing an appropriate system for gas production experiments will depend upon several factors. For example, the available resources (finance, space and time), the purpose of the studies (i.e. the aim of the experimental work) and the number of experiments planned; the manual system may be cheaper in the short term but if numerous gas production experiments are planned it may become more expensive because of the high labour costs. Due to its simplicity, however, the manual pressure transducer technique is more versatile (adaptable) than the automated systems.

2.1.5 Near infrared reflectance spectroscopy (NIR) for estimating digestibility

The application of near infrared reflectance spectroscopy (NIR) to forage analysis was first reported by Norris *et al.* (1976). The technique is explained in detail by Murray (1993) and Schenk and Westerhaus (1994). The procedure involves exposing a feed sample to near infrared radiation at different wavelengths. The feed sample absorbs some of the radiation (primarily through its C - H, N - H and O - H bonds), whilst that which is not absorbed, is reflected back to the spectrophotometer to produce a reflectance spectrum.

Originally organic matter digestibility (OMD) was predicted from NIR analysis by estimating the chemical composition of the sample by a multivariate mathematical modelling process based on a set of reference samples which had been analysed by normal chemical methods (Baker *et al.*, 1994). However, as the error associated with

the prediction was composed of both the error associated with the NIR prediction of the chemical composition of the feed (in particular the modified acid-detergent fibre (MADF) content) and that from the prediction of OMD from the chemical constituents the accuracy of the prediction was compromised. In order to improve the prediction of OMD from NIR analysis Barber *et al.* (1990) developed a prediction equation from multivariate linear regression analysis of the *in vivo* OMD of 122 silage samples with their NIR reflectance spectrums. A further 48 silage samples with known *in vivo* OMD were then analysed and their OMD predicted from the equation for validation purposes. [The importance of using an independent test set to validate NIR prediction equations has been discussed in detail by Westerhaus (1985) and Murray (1986).] Since this time, several other authors have also developed prediction equations for estimating the OMD of silages from NIR analysis of silage samples with known *in vivo* OMD (Park *et al.*, 1997; Gordon *et al.*, 1998). In addition to chemical composition and OMD predictions NIR analysis may also be used to predict voluntary food intake of grass silages (Offer *et al.*, 1998) and rumen fermentation characteristics, for example, pH, ammonia concentration, total VFA and the molar proportions of acetic, butyric and propionic acids (Offer & Percival, 1998).

The NIR technique is quick, accurate and reproducible. In some instruments, test feeds can be analysed in their natural form, hence no processing is required either in terms of drying or grinding, and as the technique is non-destructive, the sample can be used repeatedly. As NIR is a physical rather than chemical method of analysis, it does not require frequent recalibration; sophisticated software keeping an automatic record of instrument performance (Murray, 1993). It is also environmentally safe and has great potential for use in routine feed analysis (Schenk & Westerhaus, 1994; Aufrere *et al.*, 1996).

2.1.6 Chemical composition and statistical models for estimating digestibility

The chemical composition of a feed is generally easier, quicker and cheaper to determine than determining the digestibility of a feed through either *in vitro* or *in situ* procedures (Weiss, 1994). Thus, several attempts have been made to predict *in vivo*

digestibility from the chemical composition of the feed. Empirical equations derived by regressing *in vivo* digestibility on the concentration of a particular chemical fraction, such as ADF, NDF, lignin, cellulose or CP, are the most common ways of estimating digestibility (Weiss, 1994). However multi-component and theoretically - based equations may also be used (Minson, 1982; Weiss, 1994). Multi - component equations involve regressing *in vivo* digestibility on the concentration of several chemical fractions, whilst theoretically - based equations require the use of theoretically correct coefficients, rather than coefficients derived empirically (Goering & Van Soest, 1970; Weiss, 1994).

The chemical constituents of some feeds may be significantly correlated with *in vivo* digestibility. For example, Coelho *et al.* (1988) showed that ADF was significantly correlated with *in vivo* DM digestibility ($R^2 = 0.71$) for several grasses and a grass hay. However, this is not the case for all feeds, in all processed forms, as empirical relationships only work within a sub-set of the feeds used to generate them and do not take into account the physical characteristics of feeds, animal factors nor associative effects which can all influence digestibility (Weiss, 1994). Theoretically - based equations have been designed to work equally well for all types of feed, by considering digestibility coefficients and metabolic faecal excretion, however they are still unable to account for all the factors which influence digestibility (Weiss, 1994).

2.2 The Theory of Gas Production

2.2.1 Introduction

The rate of degradation of a feedstuff is one of several parameters which govern feed intake by ruminants, hence it is an important factor in ruminant nutrition (Demeyer, 1981). Traditionally, rates of degradation, have been measured using the *in situ* bag technique (Quin *et al.*, 1938; Johnson, 1966; Mehrez & Orskov, 1977). However *in vitro* techniques based on gas production, which are relatively simple and allow large numbers of samples to be assessed at one time, have become increasingly popular over the past few years. The use of gas production to evaluate the rate and extent of digestion of ruminant feeds was first described by Menke *et al.* in 1979 (section 2.1.4.3). Following the development of the Menke technique, gas production has become popular as a tool for characterising feeds and investigating the kinetics of rumen fermentation. Several different techniques for measuring gas production have been developed using pressure transducers, pressure sensitive switches and computer interfaces (Beuvink *et al.*, 1992; Pell & Schofield, 1993; Theodorou *et al.*, 1994; Davies *et al.*, 1995; Cone *et al.*, 1996). In addition to innovations in equipment design which make the technique more precise, automation of several of these systems (Beuvink *et al.*, 1992; Pell & Schofield, 1993; Davies *et al.*, 1995; Cone *et al.*, 1996) has reduced the labour involved with recording gas production manually (where gas production needs to be recorded at frequent intervals throughout the fermentation period - day and night), making the technique useful as a routine laboratory procedure.

During a typical gas production experiment, where substrate is inoculated with rumen fluid and fermented in a predominately bicarbonate buffered culture medium, gas is produced from both the fermentation of the substrate and indirectly, via acidification of the bicarbonate buffer with fermentation end-products, volatile fatty acids. As the feed particles are fermented, gas which is predominately carbon dioxide and methane, is liberated into the head-space above the liquid culture where its volume is measured. In order for the procedure to be of value, the rate and extent of gas production must be related to the rate and extent of substrate degradation (Theodorou

et al., 1994 & 1995). Although the volume of gas produced in batch culture is relatively simple to measure, the underlying processes that give rise to the gas are complex and not well understood. There is therefore concern about what is actually being measured in gas production studies and how this relates to the digestion process in the animal. This chapter provides an account of the chemical and physical principles associated with the use of gas production measurements in ruminant nutrition and their importance in gas production studies. Although much of the information regarding the behaviour of gases is available in various text books, for example, Morris (1983) and Atkins and Clugston (1986), it is essential to the nutritionist wishing to use gas production as a method of feed evaluation to have a basic understanding of how gases behave. Hence, the principles underlying the behaviour of gases have been reviewed with special emphasis on how the characteristic behaviour of gases will affect gas production measurements in terms of techniques for feed evaluation.

2.2.2 The nature of gases

In order to study rumen fermentation via the production of gas (CO_2, CH_4 and H_2) it is important to understand the nature and properties of the gaseous state. The gaseous state is considered to be the simplest of the three states of matter (solid, liquid and gas). It differs from matter in the solid or liquid state in that it has no intrinsic volume, that is, a gas will fully occupy any space into which it is introduced (Morris, 1983). There are three characteristic properties of gases; (1) their response to pressure, (2) their response to temperature and (3) their dependence on amounts. These properties are described by the Gas Laws, which accurately describe the behaviour of an 'ideal' gas. An ideal gas is a theoretical gas consisting of identical molecules of infinite size, which are in constant, random motion and do not interact with each other. However molecular interactions do occur in 'real' (actual) gases, such as hydrogen, oxygen, carbon dioxide and methane. While at low densities and high temperatures real gases will behave like an 'ideal' gas, at high densities and low temperatures their behaviour will deviate from the ideal (see section 2.2.5).

2.2.3 The gas laws

2.2.3.1 Boyle's Law (response to pressure)

Boyle's Law, formulated in 1662, describes the effect of changing volume on the pressure of a gas. By measuring the volume of a sample of gas subjected to different pressures Robert Boyle concluded that the volume (V) of a given amount of gas at constant temperature (T) is inversely proportional to the applied pressure (P) (Figure 2.2.1).

$$\text{Boyle's Law: } V \alpha 1 / P \qquad \text{(equation 2.3)}$$

$$\text{or} \quad PV = \text{constant (temperature and mass constant)}$$

This relationship provides a simple means of predicting the volume of a gas subjected to a certain pressure, and, although 'real' gases do deviate from this law, the deviations from predictions are only important when the gas is dense or cold (see section 2.2.5). As the density of any gas is reduced, so Boyle's Law is obeyed with increasing accuracy (Atkins & Clugston, 1986).

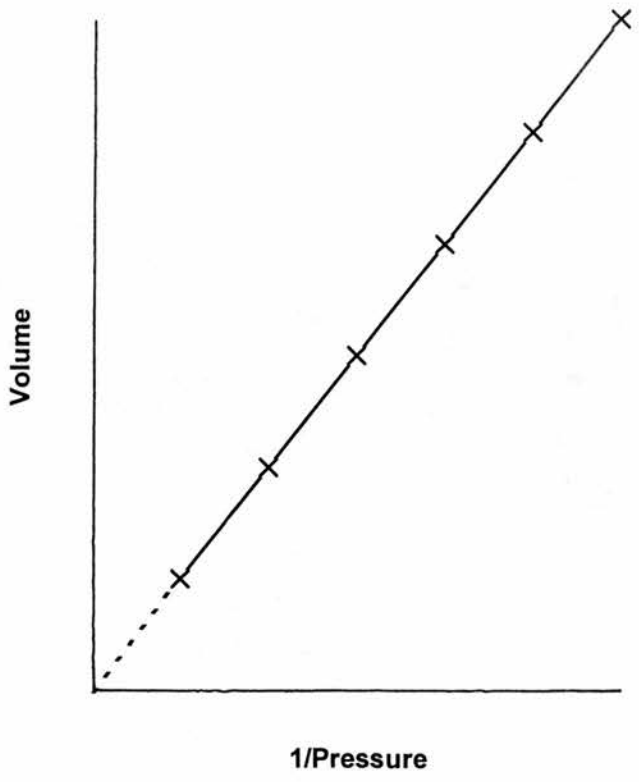
2.2.3.2 Charles' Law (response to temperature)

Charles' (or Gay-Lussac's) Law, was described in 1787, and states that at constant pressure and for a fixed mass of gas, the volume is proportional to the temperature (Figure 2.2.2). It therefore follows that, at a sufficiently low temperature, a gas should occupy nil volume. The temperature at which an 'ideal' gas will occupy nil volume is known as 'absolute zero' and corresponds to -273.15 °C or 0 K (where K represents degrees on the Kelvin temperature scale). Hence;

$$\text{Charles' Law: } V \alpha T \text{ (pressure and mass constant)} \qquad \text{(equation 2.4)}$$

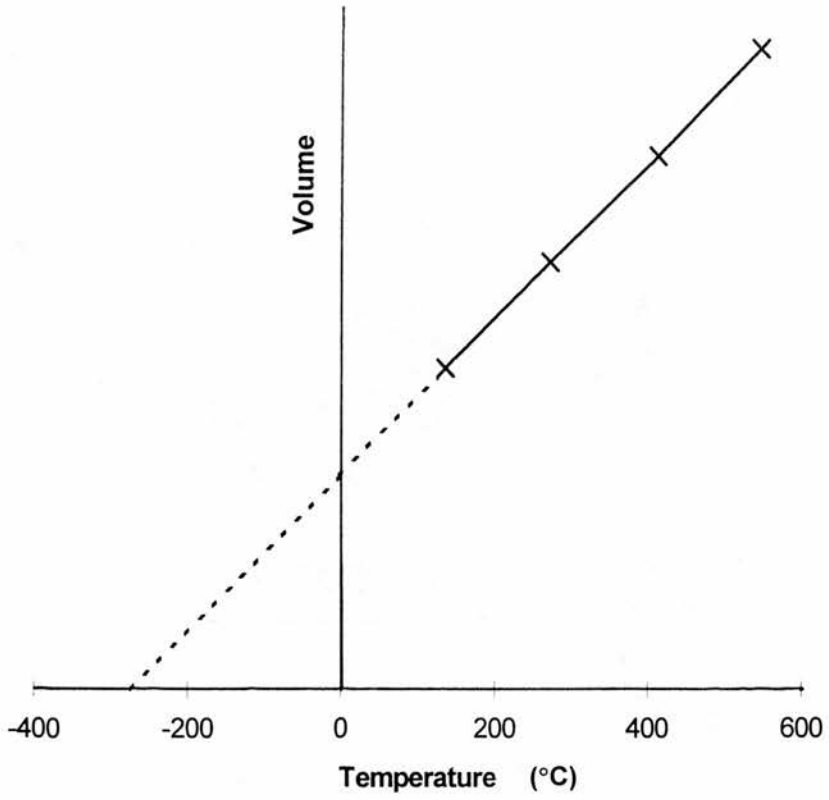
Like Boyle's Law, Charles' Law will be obeyed with increasing accuracy the more 'ideal' the gas.

Figure 2.2.1 The relationship between pressure and volume of a gas (Boyle's Law)



The volume of a given amount of gas is inversely proportional to the pressure of that gas at constant temperature (Boyle's Law). (Adapted from Atkins & Clugston, 1986).

Figure 2.2.2 The relationship between temperature and volume of a gas (Charles' Law)



At constant pressure, and for a fixed amount of gas the volume is proportional to the temperature (Charles' Law). (Adapted from Atkins & Clugston, 1986).

2.2.3.3 Avogadro's Law (dependence on amount)

Avogadro's Law describes the third characteristic of gases - their dependence on amount. Avogadro's Law states that at constant temperature and pressure, equal amounts of gases will occupy the same volume. Hence, the volume occupied by one mole of gas, the molar volume, will be the same for one mole of any other gas at the same temperature and pressure. Thus, for a gas of molar volume V_m, where the amount of substance of gas is n, the volume of gas is given by:

$$V = nV_m \qquad \text{(equation 2.5)}$$

The number of particles in one mole of gas is called Avogadro's constant (L) and has the value 6.022×10^{23} mol^{-1}. As with the other two gas laws, Avogadro's law does not hold true for all gases under all conditions. The law is useful however, in that it describes the behaviour of gases under normal conditions.

2.2.3.4 The Ideal Gas Law

The above laws may be combined together to give a single equation, the 'ideal' gas equation, which enables the state of a gas to be predicted under any conditions:

Boyle's Law	Charles' Law	Avogadro's Law
$PV = \text{constant}$	$V \propto T$	$V \propto n$

$$\Downarrow$$

$$PV \propto nT$$

$$\Downarrow$$

$$PV = nRT \qquad \text{(equation 2.6)}$$

Where n is the amount of gas, T is the temperature and R is the molar gas constant. R is equal to 8.314 $JK^{-1}mol^{-1}$ and has the same value whatever the nature of the gas (Atkins & Clugston, 1986).

Values for standard temperature and pressure (s.t.p) have been adopted to make the comparison of gases easier (Morris, 1983; Atkins & Clugston, 1986). S.t.p is defined as 0 °C and 1 atmosphere (273.15 K and 101.325 kPa, respectively). Under these conditions, 1 mole of any gas will occupy 22.4 litres (Atkins, 1992). Therefore at 39 °C, the temperature of the rumen and that used in gas production studies, the volume of one mole of gas will be 25.6 litres, that is;

$$P_1V_1 / T_1 = P_2V_2 / T_2 \qquad \text{(equation 2.7)}$$

$$1 \times 22.4 / 273.15 = 1 \times V_2 / 312.15$$

$$273.15\ V_2 = 6992.16$$

$$V_2 = 25.6 \text{ litres}$$

This is important, as the conversion of gas volume readings to s.t.p has generally not been adopted in gas production studies.

2.2.4 Mixtures of Gases

During the fermentation of a substrate by ruminal micro-organisms, several gases are produced, including carbon dioxide, methane and hydrogen. Accumulation of the gas over a liquid (the culture medium) produces an additional component (to the mixture in the head-space) in the form of water vapour. Hence, during *in vitro* gas production studies a mixture of gases is measured as opposed to a single gas, and it is therefore important to know how gas mixtures influence gas production measurements.

2.2.4.1 Dalton's Law of Partial Pressures

In a mixture of gases, the term partial pressure refers to the pressure exerted by one component of the gas mixture. Dalton's law of partial pressures was derived experimentally in 1801 and describes the pressure exerted by a mixture of gases. Dalton's law states that the total pressure observed for a mixture of gases is equal to the sum of the pressures that each individual gas would exert if it alone occupied the container at the same temperature.

$$P_t = P_A + P_B + \ldots\ldots P_i \qquad \text{(equation 2.8)}$$

where P_t is the total pressure and P_A, P_B and P_i are the pressures exerted by gases A, B and i, respectively.

For Dalton's law to hold true, individual gases in a mixture must behave independently and must not be affected by the presence of the other gases. In practice, the law is obeyed where the particles of a gas mixture are so diffuse as to allow them to act completely independently.
From the ideal gas law it follows that

$$P_t = (n_A RT / V) + (n_B RT / V) + \ldots\ldots (n_i RT / V) \qquad \text{(equation 2.9)}$$

where n_A,n_B and n_i are the amounts of the individual gases.
Using the mole fractions x_A and x_B

$$x_A = n_A/n \qquad \text{and} \qquad x_B = n_B/n \qquad \text{(equation 2.10)}$$

it is possible to calculate the partial pressure of each component gas, given the composition of the mixture and the total pressure:

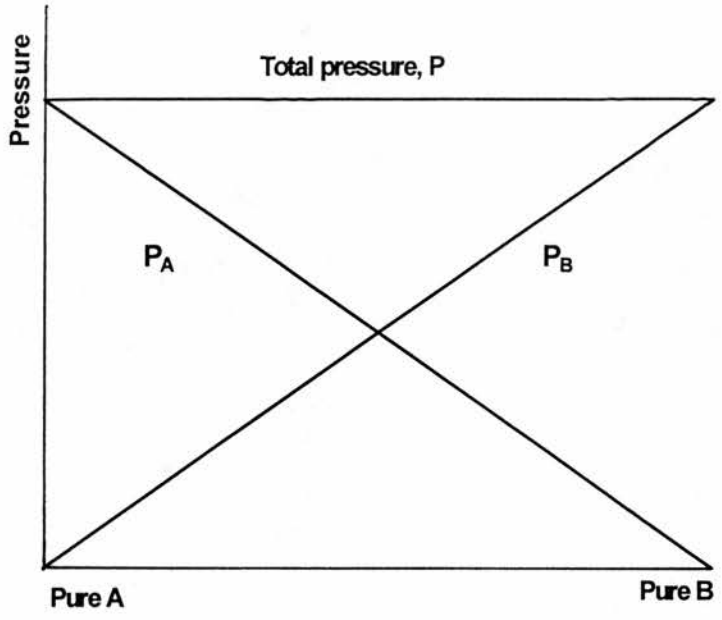
$$P_A = x_A P \qquad \text{and} \qquad P_B = x_B P \qquad \text{(equation 2.11)}$$

Figure 2.2.3 illustrates the dependence of partial pressure on the composition of the mixture.

2.2.5 The kinetic theory of gases

As well as experimental derivation, the gas laws can also be accounted for by the kinetic theory of gases - a collection of ideas which consider pressure and temperature to be a result of molecular motion. Temperature is considered to be the energy of the molecules whilst pressure is viewed as the result of gas molecules

Figure 2.2.3 Dalton's law of partial pressures; total pressure is equal to the sum of the individual pressures of gases A and B.



The total pressure exerted by a mixture of gases, A and B, is equal to the sum of the individual pressures of gases A and B, when A and B behave independently and are not affected by the presence of the other (Dalton's law of partial pressure). (Adapted from Atkins & Clugston, 1986).

colliding with the container walls where they exert a force (pressure is force per unit area). The collisions are so frequent that the walls experience virtually constant force, and hence a steady pressure. In order for the theory to be valid, three assumptions are made: (1) a gas consists of identical molecules in constant, random motion; (2) the size of the molecules is negligible compared to the average distance travelled between collisions (between themselves and with the walls of the container); (3) the molecules do not interact with each other, except during collisions which are perfectly elastic (that is, the kinetic energy of the molecules is conserved when they collide).

Under conditions of low density these assumptions can be applied to most real gases, however increasing density causes deviations from the theory.

From this theory it is possible to derive the following equation:

$$PV = {}^{1}/_{3}\, Nmu^2 \qquad \text{(equation 2.12)}$$

where V is the volume of the container, m is the mass of the molecules, N is the number of molecules present and u^2 is their mean square speed (the average value of the squares of the speeds of the molecules). This expression is derived from Newton's second law of motion, which states that force is equal to the rate at which the linear momentum (mass x velocity) of a particle changes on collision (Atkins, 1992). It is similar in form to the 'ideal' gas equation and at constant temperature and for a fixed amount of molecules, the kinetic theory would lead to 'PV = constant' which is Boyle's law (Atkins & Clugston, 1986).

2.2.5.1 The speed of gas molecules

According to the kinetic theory of gases, pressure is considered to be the force resulting from gas molecules colliding with each other and with the walls of the container vessel. Thus the larger the number of molecules present, the greater their mass and the faster their velocity, the greater the resulting pressure.

By rearranging the equation $PV = {}^1/_3\ Nmu^2$, the kinetic theory of gases can be used to calculate the average speed of gas molecules (Atkins, 1992):

$$PV = {}^1/_3\ Nmu^2$$

$$\text{to } u = \sqrt{(3PV / Nm)} \qquad \text{(equation 2.13)}$$

If N equals Avogadro's number then Nm will be equal to M, the molar mass, and the equation becomes:

$$u = \sqrt{(3PV / M)} \qquad \text{(equation 2.14)}$$

Substituting the ideal gas law, PV = RT (for one mole of ideal gas), gives the final equation for calculating the root mean square speed of gas molecules:

$$u = \sqrt{(3RT / M)} \qquad \text{(equation 2.15)}$$

From this equation it is clear that, the root mean square speeds of the gas molecules will increase as the temperature rises, and that larger molecules will generally move more slowly than smaller molecules.
Thus for gases accumulating in the head-space of a culture bottle during gas production studies, the root mean square speeds of the gas molecules are given as:

(1) carbon dioxide

(The bottles are incubated at 39 °C therefore T = 312 K)

$$u = \sqrt{3 \times (8.314\ J\ K^{-1}\ mol^{-1}) \times (312\ K) / 44.0 \times 10^{-3}\ kg\ mol^{-1}}$$

$$= 420\ ms^{-1} \text{ (approximately 940 m.p.h)}$$

(2) methane

$$u = \sqrt{3 \times (8.314\ J\ K^{-1}\ mol^{-1}) \times (312\ K) / 16.0 \times 10^{-3}\ kg\ mol^{-1}}$$

$$= 697\ ms^{-1} \text{ (approximately 1560 m.p.h)}$$

(3) hydrogen

$$u = \sqrt{3 \times (8.314 \text{ J K}^{-1} \text{ mol}^{-1}) \times (312 \text{ K}) / 2.0 \times 10^{-3} \text{ kg mol}^{-1}}$$

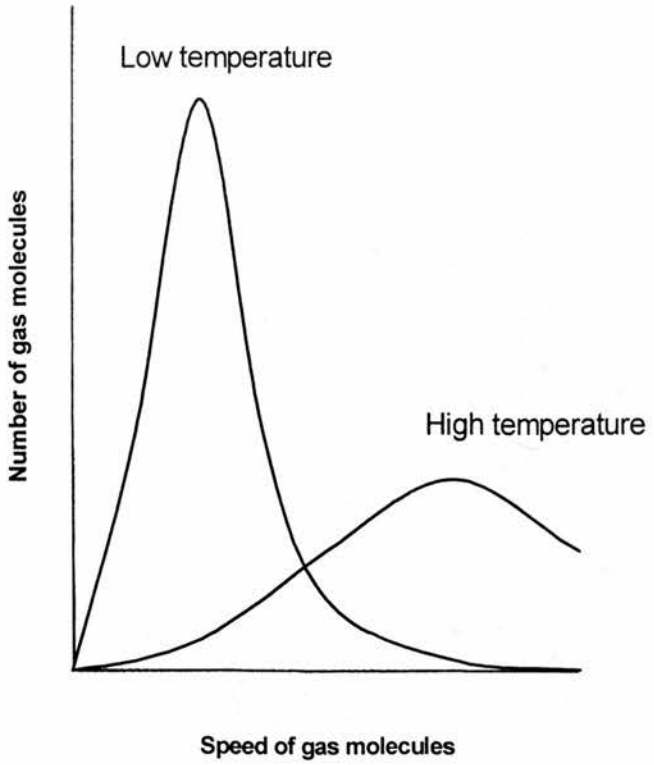
$$= 1972 \text{ ms}^{-1} \text{ (approximately 4415 m.p.h)}$$

2.2.5.2 The Maxwell - Boltzmann distribution

The speed at which the molecules of a gas are travelling is constantly changing due to collisions between molecules and between molecules and the wall of the containing vessel. Therefore at any given time, the molecules of a gas are travelling at a range of speeds. This is known as the Maxwell - Boltzmann distribution, with the largest number of molecules travelling around the mean square speed and a smaller number travelling either faster or slower than the mean.

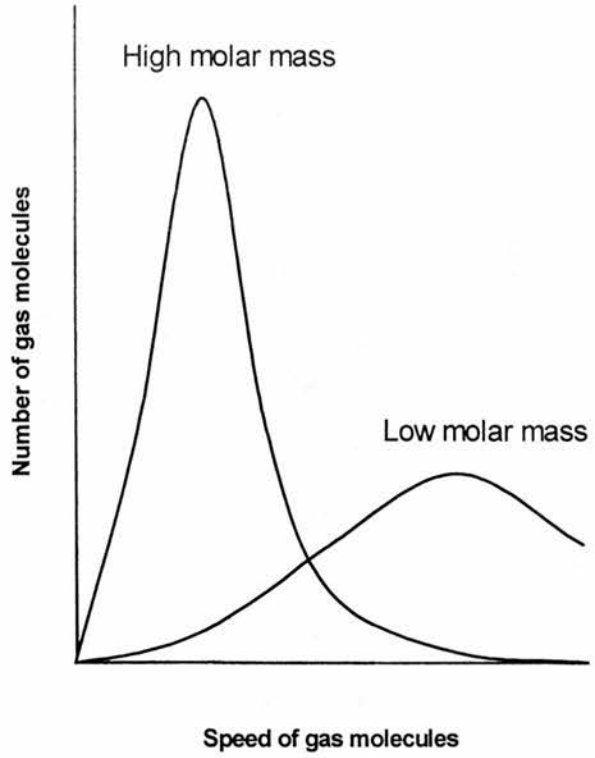
The distribution of speeds is influenced by both temperature and the molar mass of the gas molecules (Figures 2.2.4 and 2.2.5, respectively). As temperature increases the fraction of molecules with very high speeds increases due to molecules in the tail of the distribution reaching higher speeds as a result of the input of energy. Molecules with a high molar mass have a narrower distribution of speed with most travelling at speeds close to the root mean square speed. For molecules of low molar mass the distribution is wider and a significant fraction of the molecules may be travelling faster than the root mean square speed of the sample (Atkins, 1992).

Figure 2.2.4 The effect of temperature on the Maxwell - Boltzmann distribution of molecular speeds.



As temperature increases the fraction of molecules with high speeds increases, due to the input of energy. (Adapted from Atkins, 1992).

Figure 2.2.5 The effect of molecular mass on the Maxwell - Boltzmann distribution of molecular speeds.



The speed of molecules with high molar mass has a narrow distribution with most molecules travelling with speeds close to the root mean square speed of the molecules. For molecules with low molar mass the distribution of speed is much wider with more molecules travelling faster than their root mean square speed. (Adapted from Atkins, 1992).

2.2.5.3 Molecular collisions

The average distance that a molecule travels between collisions is called the mean free path (λ). In a liquid, where the molecules are close together, the mean free path is generally smaller than the diameter of the molecule itself and thus collisions occur when molecules move only a fraction of a diameter. In gases, however, the mean free path is much larger (often several hundred molecular diameters) making collisions much less frequent (Atkins, 1992). The mean free path (λ) and the frequency at

which molecules collide (z) can both be calculated from the kinetic theory of gases using the equations:

$$\lambda = RT / \sqrt{2}L\sigma P \qquad z = \sqrt{2}L\sigma u^2 P / RT \qquad \text{(equation 2.16)}$$

where R is the gas constant, T is temperature (K), L is Avogadro's constant, σ is the collision cross section of the molecules (πd^2), u^2 is the root mean square speed of the molecules (ms^{-1}) and P is the pressure (Pa) (Atkins & Clugston, 1986). From equation 2.16, values for λ for the fermentation gases, CO_2, H_2 and CH_4 at 39 °C are 58, 66 and 113 nm, respectively with corresponding values for z of 7.2, 10.5 and 17.5 billion collisions per second. Atkins (1992) described four important points to note from equation 2.16; (1) the average distance travelled by molecules between collisions is dependent upon pressure ($\lambda \alpha 1 / P$), that is, the mean free path decreases as pressure increases. This is due to a greater number of molecules occupying a given space at increased pressures, therefore each molecule has less distance to travel before it collides with another molecule. (2) the frequency of collisions increases with the pressure of the gas ($z \alpha P$), again as the number of molecules occupying a given space increases at increased pressures, a molecule will take less time to travel to another molecule, thus the frequency of collisions increases. (3) the mean free path is shorter for molecules with large collision cross-sections ($\lambda \alpha 1 / \sigma$), as larger molecules will occupy a larger volume in a given space. For example, at the same temperature and pressure, the collision cross-section of CO_2 is about twice that of a H_2 molecule, thus its mean free path is half that of H_2. (4) the collision frequency increases as the average speed of the molecules increases ($z \alpha u^2$). Thus, for molecules with a similar collision-section such as CO_2 and CH_4 where σ is 0.52 and 0.46 nm^2 respectively, the heavier CO_2 molecules have a lower collision frequency (z) than the lighter methane molecules.

2.2.6 Real gases

For the ideal gas $PV_m / RT = 1$ (where V_m is the molar volume). Experimental measurements with real gases can show striking differences from the way gases are

supposed to behave according to the 'ideal' gas equation. This is particularly obvious at low temperatures and high pressures. Deviations from the 'ideal' gas laws are due to the fact that molecules do interact with each other under certain conditions of temperature and pressure.

2.2.6.1 Intermolecular forces

In an ideal gas, molecules do not interact other than in perfectly elastic collisions; however in real gases, molecules do interact. These interactions take two forms: (1) intermolecular attraction (when molecules are far apart they tend to attract each other) and (2) intermolecular repulsion (molecules in contact tend to repel each other).

At high temperatures and low pressures, gas molecules have sufficient energy to escape the attraction of others as intermolecular forces are weak. Thus they tend to obey the 'ideal' gas laws. However, under conditions of low temperature and high pressure, intermolecular forces are stronger and molecules do not have sufficient energy to escape attraction or repulsion, resulting in deviations from the 'ideal' behaviour.

Low temperatures and high pressures are not problematic in the PTT because the bottles are incubated at 39 °C and the gas is removed from the head-space at frequent intervals throughout the incubation. This latter process is done more frequently during the initial fermentation period when gas production is most rapid.

2.2.6.2 The critical temperature

The critical temperature is the temperature at which the density of a gas equals that of its liquid. Below this temperature a gas can be condensed to it's liquid form by the application of pressure, whereby molecules are forced together so that they attract each other and cohere. However, at and above the critical temperature a gas cannot be condensed to it's liquid form by the application of pressure. Table 2.2.1 shows the critical temperature of several gases.

The critical temperatures for carbon dioxide, methane and hydrogen are all below 39 °C (312 K), the temperature used in gas production studies. Therefore, problems in recording gas production due to liquefaction of gases from the head-space of the serum bottles will not occur.

Table 2.2.1 The critical temperature of certain gases

Gas	Critical temperature (°C)
Hydrogen (H_2)	-240
Oxygen (O_2)	-118
Water (H_2O)	374
Nitrogen (N_2)	-147
Ammonia (NH_3)	132
Carbon dioxide (CO_2)	31
Methane (CH_4)	-83
Carbon tetrachloride (CCl_4)	283

The critical temperature is the temperature at which the density of a gas equals that of its liquid. Below this temperature a gas can be condensed to its liquid form by the application of pressure. At, and above, the critical temperature a gas cannot be condensed to its liquid form by the application of pressure.

2.2.6.3 Equations of state

Equations of state have been developed to adapt the ideal gas equation in order to better describe real gases. However, the use of these equations should be unnecessary, in terms of gas production studies, since the conditions under which gases accumulate ensure that the gases behave as if they were 'ideal'. In summary they are:

The Virial equation of state: This is an empirical equation proposed by Kamerlingh - Onnes in 1901. It uses a large number of constants in an attempt to precisely describe the behaviour of gases:

$$PV_m / RT = 1 + (B / V_m) + (C / V_m^2) + ... \qquad \text{(equation 2.17)}$$

where P is the pressure, V_m is the molar volume, R is the gas constant, T is the temperature (K) and B and C are empirical coefficients.
The empirical coefficients B, C , etc., are known as the virial coefficients and vary from gas to gas. They are obtained from measurements of PV_m / RT over a range of pressures.

For gases where $PV_m / RT > 1$, B must be positive, but where $PV_m / RT < 1$, B is negative until the term C / V_m^2 becomes large, at high pressures (when V_m^2 is small). At very low pressures, when the molar volume is large, the terms B / V_m and C / V_m^2 will be small, and hence the equation tends to the ideal gas equation;

$$PV_m / RT \rightarrow 1 \qquad \text{as} \qquad V_m \rightarrow \infty \qquad \text{when} \quad P \rightarrow 0 \qquad \text{(equation 2.18)}$$

The van der Waals equation of state: This equation was proposed in 1873 by Johannes van der Waals and is one of the most commonly used equations to predict the non-ideal behaviour of gases. The equation involves two modifications of the 'ideal' gas equation in order to describe 'real' gases. Firstly, gas molecules have a finite size and hence they occupy space, therefore the volume in the ideal gas equation needs to be corrected. If V is the volume available for the 'ideal' gas to move within, then in order to correct for a 'real' gas the volume needs to be reduced by a volume b, known as the covolume. The covolume is approximately four times the volume of the individual molecules and is proportional to the amount of substance (n) present. Hence V - nb replaces V_{ideal} in the 'ideal' gas law.

A second modification of the ideal gas equation involves correcting for the force of intermolecular attractions which occur in 'real' gases. As the 'ideal' gas law does not take into account the effect of the force required to overcome the force of intermolecular attractions, the pressure is overestimated (as the attractions act to slow

the molecules down so they strike the walls less frequently and with a lower impact). The reduction in pressure is therefore proportional to the square of the molar concentration (n^2 / V^2) and the reduction in pressure can be accounted for by adding the constant of proportionality a (an^2 / v^2):

$$(P + an^2 / V^2) (V - nb) = nRT$$
$$\text{or } (P + a / V_m^2) (V_m - b) = RT \qquad \text{(equation 2.19)}$$

The Van der Waals constants a and b, have been derived from experimental observations for several gases (Table 2.2.2).

Table 2.2.2 Van der Waals constants, a and b, for different gases (adapted from Maskill, 1990).

Gas	a litre^2 atm mole^{-2}	b litre mole^{-1}	*Percent difference in Vm at s.t.p
Helium	0.0341	0.0237	0.09
Hydrogen	0.2440	0.0266	0.07
Nitrogen	1.3900	0.0391	0.07
Oxygen	1.3600	0.0318	0.10
Carbon monoxide	1.4900	0.0399	0.09
Methane	2.2500	0.0428	0.20
Carbon dioxide	3.5900	0.0427	0.43
Ammonia	4.1700	0.0371	0.55

* Calculated from the Ideal Gas Law and the van der Waals Equation at 1 atmosphere and 25 °C. Where a is the constant of proportionality for the reduction in pressure, which corrects for the effects of attractions in 'real' gases and b is the proportionality constant between the reduction in volume and the amount of molecules present in the container, hence accounting for the molecule size of 'real' gases.

2.2.7 Some physical properties of gases and liquids

2.2.7.1 Introduction

During gas production studies, the mixture of gases produced from the fermentation of a substrate are collected over a liquid culture medium. It is therefore important to understand what effect the liquid will have on the gas mixture.

2.2.7.2 Diffusion and effusion of gases

Diffusion is the movement of particles from an area of high concentration to an area of low concentration, resulting in the establishment of homogeneity. Whilst, effusion is the escape of molecules through a small hole in a confining wall, for example, gas escaping via a puncture in a tyre.

The rates of diffusion and effusion depend upon the speed of the gas molecules, and are therefore affected by both temperature and molar mass. An increase in temperature increases the speed with which the molecules travel and therefore increases the rates of diffusion and effusion. Molecular speeds decrease with increasing molar mass so that the larger the molecular mass the slower the rate. The effect of molar mass is complicated in terms of diffusion where several gases may be in motion, but for effusion where the movement of a single gas is involved it is much simpler. Based on experimental observations, Graham (1833) proposed the following Law to describe the dependence of the rate of effusion of a gas on its molar mass:

Graham's Law of effusion: at a given pressure and temperature, the rate of effusion of a gas is inversely proportional to the square root of its molar mass.

Using Graham's Law, an unknown gas can be identified by comparing its rate of effusion with that of a known gas under the same conditions of temperature and pressure to derive the molar mass of the unknown gas:

$$t_A / t_B = \text{rate}_B / \text{rate}_A = \sqrt{(M_A / M_B)} \qquad \text{(equation 2.20)}$$

where t_A is the time for a given volume of gas A to escape, t_B is the time for the same volume of gas B to escape, and M_A and M_B are the molar masses of gases A and B, respectively.

2.2.7.3 The solubility of gases

2.2.7.3.1 Bunsen absorption coefficient and Henry's Law

Gases dissolve in liquids to form solutions; all gases are, to some extent, soluble in all liquids (Chou & Chao,1992; Royce, 1992; Shah *et al.*, 1993; Yamaguchi *et al.*, 1993). An equilibrium will be established between a given volume of a liquid and an excess of gas only when the liquid is fully saturated with gas. The degree of solubility of a gas in a liquid, at fixed temperature and pressure, is given by the Bunsen absorption coefficient. This is defined as the volume of gas at s.t.p which saturates a given volume of a liquid at s.t.p., and is one of the parameters used in Henry's Law.

Henry's law states that the mass of any gas which will dissolve in a given volume of liquid is directly proportional to the pressure of the gas, at constant temperature. Therefore in a mixture of gases, each gas will behave independently, and the mass of each gas that will dissolve in a given volume of liquid will be directly proportional to its absorption coefficient and to its partial pressure in the mixture (Morris, 1983). Henry's Law is described by the equation:

$$S = K\alpha P \qquad \text{(equation 2.21)}$$

where S is the solubility of the gas, K is a constant, α is the absorption coefficient for the gas in that liquid at that temperature, and P is the partial pressure of the gas. Henry's law is applicable to gases at low pressure and therefore to dilute solutions of many real gases. However, if the dissolved gas interacts with the liquid, its solubility can be greatly enhanced resulting in deviations from Henry's law. Gases such as CO_2, H_2S, NH_3, SO_2 and HCl are all highly soluble in water because they interact with the solvent (Chang, 1981). It is because of the solubility of CO_2 in water that the culture media used in gas production studies are generally gassed for several hours

prior to inoculation. This ensures that they are saturated with CO_2. Gases such as CH_4 and H_2 have low water solubility at atmospheric pressure because they do not interact chemically with the liquid.

Problems of increasing solubility may occur in gas production systems were the bottles are not vented, for example Pell and Schofield (1993). As the pressure in the head-space of the fermentation bottles builds up more CO_2 will be forced into the culture medium, and lower volumes of gas production will be recorded. It is therefore necessary in the Pell and Schofield (1993) procedure to limit the amount of substrate fermented and to develop calibration curves (section 2.1.4.3.2) to account for any changes in solubility which occur. The necessity of applying a correction factor can be avoided when the cultures are vented at regular intervals throughout the incubation.

The pressure of a gas above a liquid will also affect the solubility of that gas in the liquid; when a liquid is fully saturated with a gas, equilibrium is established. At high pressure, gas will be encouraged into the liquid phase increasing the rate of solubility whereas at low pressure, the gas will tend to remain above the liquid in the gas phase. It is therefore important to standardise the reading intervals in gas production studies; longer reading intervals will produce higher pressures in the head-space compared to frequent gas readings. This is demonstrated in chapter 4 (section 4.2). Different gas production systems have different methods of recording gas production, for example, in the automated pressure evaluation system (APES) of Davies *et al.* (1995) pressure sensitive switches release the gas when a pressure of 4.5 kPa has been reached. However, in the method of Pell and Schofield (1993), bottles are not vented and the pressure in the head-space is allowed to accumulate as the gas builds up throughout the fermentation period. Thus, the gas accumulation profiles obtained from these two systems will differ, with the vented cultures producing apparently more gas, per unit of substrate degraded, than the corresponding non-vented cultures.

Apart from the nature of the gas itself, there are several other important factors that affect the solubility of a gas.

2.2.7.3.2 The nature of the liquid

The properties of the liquid into which the gas may dissolve is perhaps the most important factor affecting the solubility of the gas. Water activity [the activity of water is defined as 1.0 for pure water and decreases with increasing osmolality, for example, sea water has an activity of 0.98], pH and buffering capacity of the liquid will all play a part in influencing gas solubility (Burton, 1987; Royce, 1992; Yamaguchi *et al.*, 1993; Lowenadler & Ronner, 1994; Khan *et al.*, 1995; Meier-Schneiders *et al.*, 1995). For example, the solubility of CO_2 increases with increasing pH, increasing water activity and increasing buffering capacity (Lowenadler & Ronner, 1994). Lowenadler and Ronner (1994) found the buffering capacity of nutrient broth to have a large effect on the solubility of CO_2; the solubility of CO_2 in nutrient broth buffered with citrate - phosphate buffer (pH 7) was approximately twice that of the non-buffered nutrient broth. They proposed that this was due to the ability of HPO_4^{2-} ions to catalyse CO_2 hydration and retain HCO_3^- ions, resulting in an increase in CO_2 solubility as described by Nyiri and Lengyel (1968).

The culture media used for gas production studies are predominately bicarbonate buffered (and saturated with CO_2 prior to inoculation) but as a consequence of the utilisation of nutrients for microbial growth and the resultant production of acidic fermentation end-products, the buffering capacity will be reduced (usually accompanied by a slight pH decline dependent on media and growth conditions) during the fermentation. As the fermentation proceeds (and in the absence of other interactions), the solubility of carbon dioxide in the culture medium is therefore likely to decline, with proportionately more of the gas appearing in the head-space.

2.2.7.3.3 The effect of temperature on the solubility of gases

The rate at which a gas dissolves in a liquid depends on temperature since most gases have a positive heat of solution, the higher the temperature the lower the rate at

which a gas will dissolve (Morris, 1983; Khan *et al.*, 1995; Meier-Schneiders *et al.*, 1995). Lowenadler and Ronner (1994) have demonstrated the effect of temperature on the solubility of CO_2 in nutrient broth with 1.1 mmol CO_2 in the head-space of closed injection bottles. They measured a drop in solubility from 7 μmol ml^{-1} at 4 °C to 4.8 μmol ml^{-1} at 22 °C.

2.2.7.3.4 The effect of surface area and gas:liquid ratio on gas solubility

The rate of solution of a gas is affected by the surface area of the gas - liquid interface; large surface areas allow more gaseous exchange between phases than small surface areas (Morris, 1983; Paul & Fincke, 1989; Meier-Schneiders *et al.*, 1995). The ratio of head-space volume to culture medium volume has also been shown to affect gas solubility. Lowenadler and Ronner (1994) investigated several different head-space to volume ratios (11:1, 1:1, 1:5) using either 20 or 100 % CO_2 in the head-space and found that the solubility of CO_2 increased as head-space to media volume ratio increased (Table 2.2.3).

Table 2.2.3 The solubility of CO_2 (μmol ml^{-1}) in different head-space to media volume ratios (Lowenadler & Ronner, 1994).

CO_2 in head-space	Head-space : media volume ratio		
(%)	11:1	1:1	1:5
20	6.0	4.0	1.7
100	26.0	12.5	3.5

The solubility of CO_2 in the culture medium used by Lowenadler and Ronner (1994), increased as the head-space to media volume ratio increased.

As surface area and the head-space to media volume ratios will be constant within a gas production study, problems are only encountered when comparing between different techniques. For example, the PTT (Theodorou *et al.*, 1994) uses 160 ml bottles filled with 100 ml of medium, whereas the Menke technique (Menke *et al.*, 1979) involves 100 ml syringes filled with 30 ml of liquid. The head-space in the bottles is fixed whereas the plunger in the syringe is displaced as more gas is

produced thereby increasing the head-space. The surface area of the liquid/ gas interface also differs being much larger in the case of the bottles compared to the syringes. Hence, even when conditions are kept as similar as possible between techniques, differences in gas production may still be observed (see chapter 7).

2.2.7.3.5 Vapour pressure

The presence of a liquid / gas interface provides the opportunity for the interchange of molecules between phases. When a liquid is introduced into a closed vessel, the rate at which molecules evaporate from the liquid will, at first, exceed the rate at which they recondense from the gaseous to the liquid phase until the partial pressure exerted by the vapour molecules results in the establishment of equilibrium (Morris, 1983).

The partial pressure of its vapour at this equilibrium is characteristic of a liquid at a given temperature and external pressure, and is known as its vapour pressure. Therefore, when the gas pressure in a head-space above a fermenting culture is measured, part of the total pressure will be due to the vapour pressure of the culture medium.

2.2.8 Biological and chemical factors influencing gas production

2.2.8.1 Microbial growth and fermentation stoichiometry

In the rumen, carbohydrate is fermented by rumen micro-organisms to produce VFA and gas (CO_2, CH_4 and H_2). Acetic (Ac), propionic (Pr) and butyric (Bu) acids constitute the majority of the VFA produced. Thus, when an energy yielding substrate in a culture medium which is predominately bicarbonate-buffered is inoculated with rumen fluid, the substrate will be converted to fermentation end products, typically the VFA (Ac, Pr and Bu) and gas (CO_2 and H_2). The conversion of the substrate to products is fundamental in energy metabolism, as it results in the production of adenosine tri-phosphate (ATP), necessary for the maintenance and growth of microbial cells. The utilisation of one mole of hexose by primary fermentation, via the Embden-Meyerhof or glycolytic pathway, results in a net yield

of two moles of ATP. Several of the end-products from primary fermentation contain sufficient energy, however, to make them useful as substrates in a subsequent or 'secondary' fermentation. For example, the process of methanogenesis which permits the methanogenic bacteria to derive energy for maintenance and growth from the conversion of CO_2 and H_2 to CH_4 (Wolin, 1975). This process results in a net energy loss to the animal, but is essential for maintaining an effective redox balance in the rumen. Subject to rate of passage, methanogenesis ensures that substrates are digested to their maximum extent in the rumen (Hungate, 1966).

The formation of acetic and butyric acids generally result in the production of gas. When acetic acid is produced by the oxidative decarboxylation of pyruvate, 2 moles of CO_2 and 4 moles of H_2 are produced for every mole of glucose degraded (Hungate, 1966):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \text{(equation 2.22)}$$

The formation of butyric acid generally occurs through the condensation of 2 molecules of acetic acid to form acetoacetic acid, which is then reduced to butyric acid with four atoms of H, producing 2 moles of CO_2 and 2 moles of H_2 (Hungate, 1966):

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad \text{(equation 2.23)}$$

However, the pathway by which propionic acid is derived does not produce gas but instead utilises H (Hungate, 1966):

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \qquad \text{(equation 2.24)}$$

CO_2 and H_2 produced by these reactions can combine to form methane (Hungate, 1966):

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \text{(equation 2.25)}$$

Most of the H_2 that is produced is converted into CH_4 and VFAs, with less than one percent being detected in the gas phase (von Grabe, 1978). Production of CH_4 does not influence the amount of gas produced as the H_2 is incorporated into CH_4, and 1 mole of CH_4 replaces 1 mole of CO_2. Hence, the volume and composition of the gas produced from the fermentation of hexose will depend upon the quantity and molar proportions of the VFA produced (see section 5.1). Therefore, a shift in the pattern of fermentation which increased the proportion of acetic and butyric acids whilst decreasing that of propionic acid, would result in an apparent increase in the volume of gas per mole of hexose fermented. Conversely if propionic acid production was favoured at the expense of acetic and butyric acids, a decrease in the volume of gas produced per mole of hexose fermented would be noted.

In addition to the gas produced during the microbial fermentation of a substrate (fermentation gas) by the above pathways, carbon dioxide may also be released from the incubation buffer upon production of volatile fatty acids (acidification gas).

2.2.8.2 Buffers

Buffers are essential components of the culture media used in microbiological studies. The purpose of the buffer is to maintain the medium at the optimum pH for the desired micro-organisms. Two important points to consider when choosing a buffer are (1) the components of the buffer should not interfere with the reaction in any way (other than to stabilise pH) and (2) the pKa (the negative logarithm of the acid dissociation constant, K_a) of the acid component (at the working temperature) should be close to the desired pH (Morris, 1983). The buffer capacity of the solution is an indication of how effective the buffer will be at minimising the pH change upon addition of a standard quantity of strong acid or strong base. Solutions which contain a weak acid - conjugate base, or a weak base - conjugate acid, make the best buffers in the pH range 4 - 10 (Morris, 1983). The conjugate acid is the acid formed by the association of a base with a proton, for example, ammonia (NH_3) is a highly soluble

weak base which is capable of accepting protons to form its conjugate acid, ammonium (NH_4^+):

$$NH_3 + H^+ \leftrightarrow NH_4^+ \qquad \text{(equation 2.26)}$$

Correspondingly, a conjugate base is the base formed when an acid dissociates, for example, the acetate ion, CH_3COO^-, is the conjugate base of acetic acid CH_3COOH:

$$CH_3COOH \leftrightarrow H^+ + CH_3COO^- \qquad \text{(equation 2.27)}$$

The pH of a buffer can be calculated using the Henderson - Hasselbalch equation:

$$pH = pKa + \log [base]/[acid] \qquad \text{(equation 2.28)}$$

(where pKa is the value of its acid component)

In general, a mixture with a [base]/[acid] ratio of 1, is optimally buffered against both strong acid and strong base, and will have a pH equal to the pKa of the acid component. Mixtures with [base]/[acid] ratios between 0.1 and 10 have significant buffering capacity and their pH will be close to the pKa value of their acid component (Morris, 1983).

Media for gas production studies generally contain both a phosphate and a bicarbonate buffer. The bicarbonate buffer is a weak base - conjugate acid:

$$HCO_3^- + H^+ \leftrightarrow H_2CO_3 \qquad \text{(equation 2.29)}$$

the pKa of carbonic acid is 6.37 therefore the pH of the buffer will be approximately 6.37. The phosphate buffer (a weak acid - conjugate base) has a series of dissociation steps:

$$H_3PO_4 \leftrightarrow H^+ + H_2PO_4^- \leftrightarrow H^+ HPO_4^{2-} \leftrightarrow H^+ + PO_4^{3-} \qquad \text{(equation 2.30)}$$

The pKa values for each step are 1.96, 6.80 and 12.0 respectively. Therefore the predominant conjugate pair in gas production media will be:

$$H^+ + H_2PO_4^- \leftrightarrow H^+ HPO_4^{2-} \qquad \text{(equation 2.31)}$$

as this has a pKa value closest to the desired pH of 6.5 - 6.8 (Russell & Dombrowski, 1980).

2.2.8.3 The production of gas by acid-base titration

In addition to the gas produced from fermentation, gas will also be released from the medium upon the addition of acidic fermentation end products (VFA). The amount of gas released from the buffer by the VFA produced will depend upon the composition of the buffer. For example, the addition of 1 mole of acid to a bicarbonate buffer would be expected to produce 1 mole of gas (Steingass & Menke, 1986):

$$H^+ + HCO_3^- \leftrightarrow H_2CO_3 \leftrightarrow CO_2 + H_2O \qquad \text{(equation 2.32)}$$

However, many of the culture media routinely used in rumen microbiology contain a phosphate buffer in addition to the bicarbonate buffer. Thus, a certain proportion (relative to the proportion of phosphate buffer present) of the H^+ ions formed during fermentation will be neutralised without the formation of gas. Hence the quantity of gas released from a phosphate / bicarbonate buffer per mole of VFA added will be less than 1 mole. For example, studies using the pressure transducer technique of Theodorou *et al.* (1994) employ a bicarbonate / phosphate buffer (section 3.7) where, for every mole of VFA added, approximately 0.62 moles of carbon dioxide was released (section 5.1). Given that the VFAs are mono-basic acids with strong dissociation constants (acetic, propionic and butyric acids all have pKa values of approximately 4.8; Argenzio & Stevens, 1984), similar quantities of acidification gas were produced by acetic, propionic and butyric acids (Table 2.2.4).

Table 2.2.4 Gas produced (moles) during the formation of acetic (Ac), butyric (Bu) and propionic (Pr) acids from one mole of glucose.

VFA (moles)	*Fermentation gas*	*Acidification gas*	*Total gas*
2 Ac	2 CO_2	(2 x 0.62)	3.24 CO_2
	4 H		4.0 H
1 Bu	2 CO_2	(1 x 0.62)	2.62 CO_2
	2 H		2.0 H
2 Pr	-	(2 x 0.62)	1.24 CO_2
	-2 H		-2.0 H

During the fermentation of one mole of glucose the amount of gas produced depends upon the pathway followed, that is, in the formation of acetic, propionic or butyric acids. Gas is also produced as a result of the neutralisation of the acidic fermentation end-products by the incubation buffer (acidification gas).

Beuvink and Spoelstra (1992) found that the addition of 1 mole of VFA released 0.87 moles of gas from the buffered rumen fluid used in their studies. Table 2.2.5 summarises the moles of gas released from different media used in rumen microbiology following the addition of 1 mole of VFA. Blummel and Ørskov (1993) reported that approximately 50 % of the gas produced during the fermentation of straw came from the neutralisation of VFA by the buffer. Using this observation they found a good correlation between the predicted gas production, using the stoichiometry of Wolin (1975) (equation 2.33) together with acidification gas, and the actual gas recorded during the incubation of straw with buffered rumen fluid.

$$57.5\ (C_6H_{12}O_6) \rightarrow 65\ CH_3CO_2H + 20\ CH_3CH_2CO_2H + 15\ CH_3CH_2CH_2CO_2H + 60\ CO_2 + 35\ CH_4 + 25\ H_2O \qquad \text{(equation 2.33)}$$

However, this was based on the assumption of Steingass and Menke (1986) that, for every mole of VFA produced, one mole of gas will be released from the buffer. As this was not the case, their values of acidification gas appear to have been significantly overestimated.

Table 2.2.5 The quantity (moles) of gas released from different rumen microbiology media following the addition of 1 mole of volatile fatty acid (acetic, propionic or butyric acid).

Author	Medium	Moles of gas
Steingass & Menke, 1986	Menke *et al.*, 1979 as modified by Steingass, 1983.	1.00
Beuvink & Spoelstra, 1992	as above	0.87
Sileshi, 1994	Theodorou & Brooks, 1990	0.64
Nielsen, 1996	semi defined Lowe's medium (Lowe *et al.*, 1985)	0.64

The amount of gas produced by the addition of fermentation end-products to rumen microbiology media will vary depending upon the buffers used in the culture medium.

The interpretation of gas production profiles could be simplified if bicarbonate buffer was removed from the medium and only a phosphate buffer employed. This would eliminate the complication of CO_2 release from the medium. However, according to Pell and Schofield (1993), in order to simulate the rumen environment as closely as possible (by providing HCO_3^- ions and maintaining a pH of 6.5 - 6.8), the bicarbonate buffer is essential to the medium.

2.3 The application of models to gas production data

2.3.1 Introduction

Mathematical models are important tools for evaluating hypotheses and describing experimental results. 'A mathematical model is an equation or set of equations which represents the behaviour of a system' (France & Thornley, 1984). These models may be either static, that is when time is not a variable in the equation, or dynamic, when time is included as a variable in the equation. They may also be empirical or mechanistic (theoretical). Empirical models tend to describe the relationship between variables only, for example regression equations are empirical, whilst mechanistic models are derived from concepts concerning the nature of the system which is to be modelled. Lastly, the model may be deterministic or stochastic. Deterministic models make definite predictions for quantities whilst stochastic models include a probability distribution in order that both the quantities and the variance associated with those quantities can be predicted (France & Thornley, 1984).

The basic principle for all gas production models is that a potentially degradable substrate (S) is fermented to give a yield of gas (y) at a rate μ; where μ is the fractional rate of degradation (h^{-1}) at time t:

$$S \xrightarrow[\mu\ (h^{-1})]{} y \qquad \text{(equation 2.34)}$$

In this way, a model can be used to describe a gas production profile and it is therefore possible to compare gas production profiles from different feeds.
There are several types of model which can be used to fit curves to gas production data, for example, France *et al.* (1993), Schofield *et al.* (1994), and Groot *et al.* (1996). These models are generally based on four basic types of expression; Mitscherlich, Michaelis - Menten, logistic or Gompertz. These four curves are described in detail by France and Thornley (1984) and Ross (1987). The main requirement of a model for gas production studies is that it is adaptable in order to fit a wide range of gas production profiles (France *et al.*, 1997).

2.3.2 The model of France et al. (1993)

The France *et al.* (1993) model was derived to describe the gas production data obtained using the pressure transducer technique of Theodorou *et al.* (1994). The model was based on the simple exponential adopted by Ørskov and McDonald (1979), which is frequently applied to the *in situ* bag technique used in feed evaluation (Beuvink & Kogut, 1993). However, this model cannot be used to describe sigmoidal responses, and is therefore inappropriate for modelling gas production, which is often sigmoidal with time. In order to accommodate sigmoidal responses France *et al.* (1993) have modified the basic equation:

$$Y = A \exp[-b (t - L_T)] \qquad \text{Ørskov \& McDonald (1979)}$$

(equation 2.35)

by the addition of an extra parameter to give:

$$Y = A \{1 - \exp[-b (t - L_T) - c(\sqrt{t} - \sqrt{L_T})]\} \qquad \text{France } et\ al. \text{ (1993)}$$

(equation 2.36)

where Y is the gas production (ml) at time t (h), A is the asymptote of gas production (ml), b (h^{-1}) and c ($h^{-0.5}$) are rate constants and L_T is the lag time (h).
The fractional rate of degradation, μ (h^{-1}), can be calculated (at any time in the incubation) using the following equation:

$$\mu = b + c / 2\sqrt{t} \qquad \text{(equation 2.37)}$$

The rate constants b and c both influence the fractional rate of degradation, μ. However, whilst the influence of b is independent of time, the influence of c decreases with time. The value of c therefore influences the shape of the gas profile; if c is negative the resulting gas profile is sigmoidal, whilst a positive c indicates that the growth rate is initially faster than exponential, finally, if c is zero an exponential growth curve is described.

The extent of degradation in the rumen may also be predicted from the model using the equation:

$$E = S_o e^{-k\,LT} (1 - kI) / (S_o + U_o) \qquad \text{(equation 2.38)}$$

where S_o and U_o are the zero time quantities of the degradable and undegradable fractions of the feed component, respectively, L_T is the lag time (h), k (h^{-1}) is the passage rate constant (usually estimated at around 2 %) and I is the integral:

$$I = \int_{LT}^{\infty} \exp \{ - [(b + k) (t - L_T) + c (\sqrt{t} - \sqrt{L_T})\} \, dt \qquad \text{(equation 2.39)}$$

2.3.3 The model of Schofield et al. (1994)

Gas production profiles, obtained using the automated system of Pell and Schofield (1993), are described by the model of Schofield *et al.* (1994). Their model is more complex than that of France *et al.* (1993) because it has been developed to cope with multi-pool kinetics. It involves a modified, dual-pool, logistic equation with a single lag value:

$$V = V_{F1} \{1 + \exp(2 + 4S_1[\lambda_1 - t])\}^{-1} + V_{F2} \{1 + \exp(2 + 4S_2[\lambda_2 - t])\}^{-1} \qquad \text{(equation 2.40)}$$

where V is the gas volume at time t, V_F is the maximum gas production, S is a rate constant called the specific rate constant (S = maximum rate / maximum volume) and λ is an integration constant equivalent to a lag term (note: $\lambda_1 = \lambda_2$).

Pell *et al.* (1997) have investigated curve subtraction, or curve stripping, by subtracting the gas production from a known component of the substrate from the total gas production to estimate the gas production from the remaining component. In order for curve subtraction to be valid, several assumptions must be made: (1) the method used to extract a component from the substrate does not affect the digestion kinetics, (2) residual extractants are not toxic to the microbial population and (3) feed

components do not interact, that is, feed components do not interact to produce more or less gas than if they were incubated separately.

2.3.4 The multi phasic model of Groot et al. (1996)

Progressing from the model of Schofield *et al.* (1994), Groot *et al.* (1996) have developed a model that they believe is more flexible for modelling gas production data; especially that collected from the automated gas production system of Cone *et al.* (1994).

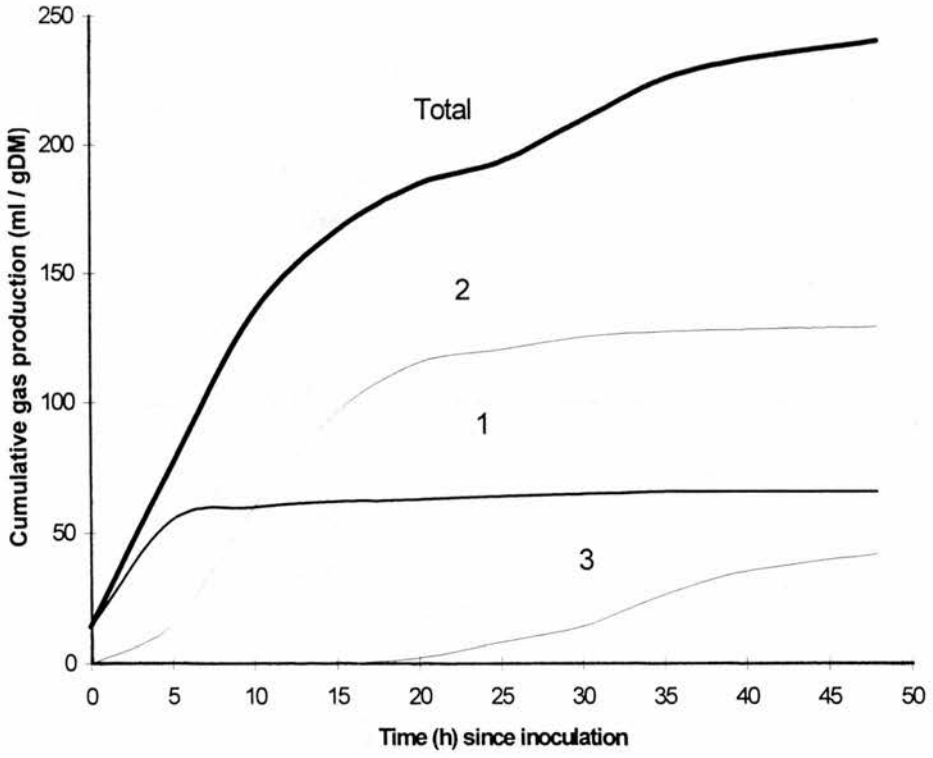
Groot *et al.* (1996) have developed a multi phasic model, based on the Michaelis - Menten equation, to describe gas production profiles:

$$\text{ml gas} = a_1 / (1 + (b_1 / t)^{c1}) + \ldots + a_n / (1 + (b_n / t)^{cn}) \qquad \text{(equation 2.41)}$$

where a is the maximal gas production (or asymptote) (ml), b is the time at which half of the maximal gas production is produced (h), c is a parameter to determine the shape of the curve, t is the incubation time (h) and n represents the number of phases (subcurves).

In general, gas production from feedstuffs can be split into three subcurves (Cone, 1997; Cone *et al.*, 1997); the first represents fermentation of the soluble fraction of a feedstuff, the second, fermentation from the non - soluble fraction whilst the third subcurve, which starts approximately 15 - 20 h after inoculation, is not related to the fermentation of the substrate itself but rather to microbial turnover (Figure 2.3.1).

Figure 2.3.1 The gas production profile from the incubation of a sample of grass in buffered rumen fluid; showing total gas production and the three subcurves according to Groot *et al.* (1996) and Cone (1995; 1997). Where subcurve 1 represents gas production from the soluble fraction; subcurve 2 is produced from the insoluble but fermentable residue and subcurve 3 represents microbial turnover. (Adapted from Cone, 1995).



The advantage of the Groot *et al.* (1996) model is its potential for identifying the fermentation of different carbohydrate pools (as determined by incubating the isolated NDF residue of the feeds) which will provide more information about complex substrates than analysis of the whole feed alone (Moss & Givens, 1997).

2.3.5 Comparison of the models used in gas production studies

A comparison of four model types by France *et al.* (1997) showed that the Mitscherlich and Michaelis - Menten based equations were significantly better ($p < 0.05$) at fitting a wider range of shapes than either the Gompertz or logistic equations and were thus better at fitting gas production profiles. The Ørskov and McDonald

(1979), France *et al.* (1993) and the monophasic form of the model proposed by Groot *et al.* (1996) were compared by Macheboeuf and Van Milgen (1997) on the basis of their ability to describe gas production profiles from green forages, grass hays and alfalfa hays. They found that the model of France *et al.* (1993) was the best in terms of predicting the gas production after 24 h fermentation, whilst the Ørskov and McDonald (1979) was the worst. The ability of three models [modified Gompertz (Beuvink & Kogut, 1993), France *et al.* (1993) and Ørskov & McDonald (1979)] to accurately describe gas production profiles for ten whole crop wheat forages were compared by Adesogan *et al.* (1997). They showed that the Ørskov & McDonald (1979) model was unable to fit the exponential part of the curve, the France *et al.* (1993) model underestimated the asymptotic phase, whilst the modified Gompertz model, which accounts for gas production from slowly and rapidly fermentable feed fractions, consistently described the data most accurately. However, the modified Gompertz model may not be the best model to use where slowly degradable feeds, with a long lag phase and slow initial degradation, are investigated (Nielsen, 1996). Model comparisons are summarised in Table 2.3.1.

Table 2.3.1 A comparison between different models in terms of their ability to describe gas production profiles.

	Model				
Substrate	France *et al.* (1993)	Beuvink & Kogut (1993)	Groot *et al.* (1996)	Ørskov & McDonald (1979)	Author
Grass hay	***		**	*	1
Alfalfa hay	***		**	*	1
Green forages	***		**	*	1
Whole crop wheat	**	***		*	2

Stars indicate the best (***) to worst (*) models for predicting gas production parameters, as determined by the various authors. The above information was obtained from (1) Macheboeuf and Van Milgen (1997) and (2) Adesogan *et al.* (1997).

Of the parameters predicted by gas production models, the rate constant(s) is perhaps the most important, often providing a better correlation with feed intake, digestibility and performance characteristics than any other single value, such as the asymptote or lag time, that describes the maximum potential degradability of forage fibre (Ørskov & Ryle, 1990). However, with the France *et al.* (1993) model, there are two rate constants. Therefore the fractional rate of degradation at the time taken to produce 50 % of the total gas production, t_{50}, is often used to compare feeds and this may be a more useful parameter to estimate degradability than the individual rate constants (see section 8.1).

Of the models described here, neither the Schofield *et al.* (1994) model nor the Groot *et al.* (1996) model allow the extent of degradation in the rumen (or caecum) to be predicted, whilst the France *et al.* (1993) model has been developed in order to permit the extent of degradation to be predicted. In this thesis the model of France *et al.* (1993) was used to fit gas production profiles principally because the model was developed specifically for the pressure transducer technique. In addition, the model of France *et al.* (1993) allows the extent of degradation in the rumen (or caecum) to be predicted, which may be useful in future work when information on the passage rate of these feeds is available.

2.4 Summary of the literature review

Several techniques are available for assessing the digestibility of animal feeds. These techniques can be divided into either *in vivo*, *in situ* or *in vitro* procedures. In order to be used in routine feed analysis the technique selected must meet several criteria; it must be rapid and simple to perform, give reliable, reproducible results and most importantly, the results must be relevant to what happens in the animal. Measurement of digestibility is based on *in vivo* experimentation in live animals. However, *in vivo* trials tend to be labour intensive, time consuming and expensive in terms of both the number of animals and the amount of feed required, making them unsuitable for routine use. *In situ* techniques are also unsuitable because they require surgically prepared animals which are expensive both to prepare and maintain, furthermore, only a few samples can be analysed at one time. *In vitro* techniques are rapid, use small quantities of feed and allow several samples to be analysed at any one time, they therefore, show the greatest scope for routine feed analysis. *In vitro* techniques which have been developed for feed evaluation include incubation with rumen fluid or cellulolytic enzymes, and near-infrared reflectance spectroscopy (NIR).

The majority of *in vitro* prediction techniques provide information on the end-point degradability of the sample only. However the rate of degradation as well as the extent of degradation can be predicted quickly and efficiently by *in vitro* gas production techniques, thus providing information on how the feed is used by the animal.

In order to use gas production techniques as a means of assessing digestive processes, it is important to understand the nature and properties of the gaseous state. As discussed in section 2.2, several factors affect the behaviour of gases. The three main factors which influence the behaviour of gases are described by their three characteristic properties; (1) their response to pressure (Boyle's Law), (2) their response to temperature (Charles' Law) and (3) their dependence on amounts (Avogadro's Law). In addition to pressure, temperature and amount of substance present, the conditions under which the gas is measured will also affect its

measurement. One of the most important points to consider during gas production studies is that the mixture of gases produced during fermentation of the substrate, are collected over a liquid culture medium. Therefore, the solubility of the gas, the nature of the liquid culture medium, the surface area of the gas / liquid interface and the ratio of gas: liquid all need to be taken into consideration.

In addition to the above factors, the properties and nature of the substrate will also influence the measurement of gas production during *in vitro* fermentation. In the rumen, carbohydrate is fermented by rumen micro-organisms to produce volatile fatty acids (VFA) and gas, the balance of which is dependent upon the nature of the substrate. Thus during incubation of a substrate in a predominately bicarbonate-buffered culture medium with rumen fluid, the substrate is fermented to VFA and gas. However, in addition to the gas produced by the fermentation itself, CO_2 will also be released from the medium upon neutralisation of the acidic fermentation end products, VFA, with the bicarbonate buffer (acidification gas). Hence, it is important to take acidification gas into consideration when comparing or interpreting gas production profiles.

Mathematical modelling of the gas production profiles obtained during the fermentation of a substrate contributes greatly to the description and interpretation of gas production profiles. Several different models have been developed in order to describe gas production profiles accurately (section 2.3). The basis for all these models is that a potentially degradable substrate (S) is fermented to give a yield of gas (y) at a rate μ; where μ is the fractional rate of degradation (h^{-1}) at time t. Of the models developed there is no particular one which consistently describes gas production profiles more accurately than the others. However, only the model of France *et al.* (1993) allows the extent of degradation in the rumen to be predicted.

2.5 The scope of this thesis

The aim of this thesis was to investigate the suitability and application of the gas production technique as a routine analytical tool in animal nutrition studies. Firstly, several of the different factors which affect the behaviour of gases were investigated in order to develop a standard protocol for the technique which minimised variation. Several physical, biological and chemical factors were examined including; head-space pressure, temperature, shaking movement, the nature of the substrate (chemical composition, particle size and drying process), the source of microbial inoculum and the apparatus used to record gas production. Secondly, two possible applications for the technique were investigated. These were (1) the prediction of digestible energy values for equine feeds and (2) the use of the technique as a screening tool to investigate the potential of feed additives.

CHAPTER 3 - GENERAL MATERIALS AND METHODS

This chapter details the general materials and methods which have been used throughout this thesis. Detailed descriptions of the experimental protocols used in the study are given in the appropriate experimental chapters.

3.1 Feedstuffs

The feedstuffs investigated throughout this thesis were oatfeed (OF), naked oats (*Avena nuda*) (NO), soya hulls (SH) and unmolassed sugar beet pulp (*Beta vulgaris*) (SB) [supplied by Dalgety Agriculture Ltd., Milton Keynes, UK] and a perennial ryegrass (*Lolium perenne*) hay (H), Italian ryegrass (*Lolium multiflorum*) (RG) and white clover (*Trifolium repens*) (WC) [IGER, Aberystwyth, UK]. All feedstuffs were freeze-dried and ground to pass through a 1mm dry mesh screen, unless otherwise stated. The chemical composition of the feeds in relation to their DM, ADF, NDF, OM and CP content is shown in Appendix 1.

3.2 Animals and their management

3.2.1 Sheep (IGER, Aberystwyth)

Two mature, rumenally - fistulated Clun forest sheep (mean weight 65 kg) were used as a source of ovine rumen digesta. The sheep were fed Italian ryegrass (*Lolium multiflorum*) hay *ad libitum*, eating approximately 1.5 kg per day, together with 0.15 kg per day of sheep coarse mix (Superstock, BOCM). Animals had free access to a clean supply of drinking water and a mineral block (Battle, Hayward & Bower Ltd., Lincoln, UK).

3.2.2 Steers (SAC, Edinburgh)

Three mature, rumenally - fistulated Hereford x Friesian steers (mean weight 850 kg) were used as sources of bovine rumen digesta. They were fed 7 kg perennial ryegrass (*Lolium perenne*) hay, divided into two equal feeds per day, and had free access to a clean supply of drinking water. Approximately 50 g of a mineral vitamin supplement (Norvite Feed Supplements, Insch, Aberdeenshire, UK) was fed daily to each animal.

3.2.3 Ponies (Edinburgh University)

Three mature, caecally - fistulated, Welsh - cross ponies (mean weight 280 kg) were used as sources of caecal digesta. All ponies received perennial ryegrass (*Lolium perenne*) hay *ad libitum*, eating approximately 5.5 kg per day, and had free access to a clean supply of drinking water. A mineral vitamin supplement (60g; Norvite Feed Supplements, Insch, Aberdeenshire, UK) was fed daily in 75 g of unmolassed sugar beet pulp (Dalgety Agriculture Ltd.) to each animal.

Equine faeces were collected from the fistulated ponies (section 5.3) or from four other ponies, fed perennial ryegrass (*Lolium perenne*) hay *ad libitum,* and housed at Bush Home Farm Stables, Edinburgh University (section 8.1).

3.2.4 Housing and maintenance

Fistulated animals were housed indoors; the steers were penned together in a pen 4 m x 13 m, whilst both sheep and ponies were kept in individual pens, measuring 1.46 m x 1.70 m and 3 m x 4 m, for sheep and ponies respectively. For all animals, the area around the fistula was washed on a regular basis and also after collection of digesta.

3.3 Sample collection

3.3.1 Ovine and bovine rumen digesta

Approximately 500 ml of rumen digesta was collected from each animal before the morning feed. Digesta was collected by removing the cap of the cannula, and relying on ruminal contractions to expel digesta through the cannula. The digesta was collected in a thermos flask, which had been pre-warmed to 39°C with hot water, and quickly transported to the laboratory for processing.

3.3.2 Equine caecal digesta

In general, approximately 400 ml of caecal digesta were collected from each pony and pooled to give a total volume of 1200 ml. The digesta was collected before the morning feed by removing the cap of the cannula and relying on caecal contractions to expel digesta into a plastic bag held over the cannula. If no contractions occurred,

the ponies were walked to facilitate contractile movement. Caecal digesta was transferred into a prewarmed (39°C) thermos flask and quickly transported to the laboratory for processing.

3.3.3 Equine faecal material

Faeces were collected within 1 h of voiding from either the same ponies that had been the source of caecal digesta (section 5.3) or from four ponies fed perennial ryegrass (*Lolium perenne*) hay *ad libitum* housed at Bush Home Farm Stables (section 8.1).

3.4 Preparation of microbial inoculum

Preparation of the microbial inoculum involved macerating the digesta in a Kenwood liquidiser (Kenwood BL300; Kenwood Ltd., Harant, Hants, UK) for 1 - 2 min followed by straining through three layers of muslin. Maceration ensured that particle-associated micro-organisms were included in the resultant inoculum. The fluid was collected in a beaker and strained a second time, to ensure all large fibre particles had been removed. Throughout the process, the fluid was continually flushed with CO_2 gas and stirred (with a magnetic stirrer and bar). The method of preparation of inoculum was the same for all sources, that is inoculum from, ovine rumen, bovine rumen, equine caecal or equine faecal samples. The inoculum was dispensed immediately after preparation.

3.5 Anaerobic Techniques

The rumen (and caecum) is an anaerobic environment, with a redox potential of -300 to -350 mV (Theodorou & France, 1993). The composition of the gas in the rumen is generally 65 % carbon dioxide, 27 % methane, 7 % nitrogen, 0.2 % hydrogen, 0.1 % hydrogen sulphide and 0.6 % oxygen (McArthur & Miltimore, 1961). It contains a large number of obligate, anaerobic micro-organisms, such as, bacteria (Bryant *et al.*, 1958; Hungate, 1966), fungi (Orpin, 1975 & 1976), protozoa (Clarke, 1964 & 1977) and bacteriophage (Adams *et al.*, 1966). These organisms are protected from the toxic effect of oxygen, which may enter the rumen with ingested plant material, by

the presence of facultative bacteria which scavenge ingested oxygen (Baldwin & Emery, 1960; Stewart *et al.*, 1988). It is also possible in grazing ruminants that the enzymes in the grazed grass can effectively remove significant quantities of oxygen from the rumen (M.K. Theodorou - personal communication).

Due to the anaerobic nature of the rumen, in gas production experiments it is important that the rumen fluid inoculum is maintained under anaerobic conditions. Anaerobic techniques for the cultivation of anaerobic rumen bacteria were first described by Hungate (1969), and have since been modified and extended to provide the routine procedures used today (Bryant, 1972; Miller & Wolin, 1974).

Oxygen can be removed from the culture media by firstly boiling (de-gassing), then 'gassing' with 100 % CO_2 or a mixture of CO_2 and N_2. With the media used in the studies reported in this thesis, gassing of the media for 3 - 5 h proved to be sufficient; boiling was not necessary. The process of boiling and gassing does not remove all traces of oxygen dissolved in the media and therefore does not lower the redox potential to a low enough value for all rumen anaerobes. Hence chemical reducing agents, such as sodium sulphide and / or L - cysteine hydrochloride, are required (Ljungdahl & Wiegel, 1986), together with a redox indicator to indicate the redox potential of the medium; the majority of anaerobic media contain the indicator resazurin (Theodorou *et al.*, 1996b). Resazurin has a redox potential of - 51 mV, and changes from blue to pink as the medium is reduced. When the redox potential falls below - 110 mV, usually after addition of a reducing agent to the gassed medium, the resazurin becomes colourless. When reoxidised, resazurin becomes pink again, but will not return to the blue colour (Ljungdahl & Wiegel, 1986).

Gassing lines were prepared from impervious butyl rubber tubing (Esco, Feltham, Middlesex, UK). Plastic T - pieces (Fisher Scientific UK, Loughborough, UK) were used to arrange the tubing to form four open ends. At each open end, a syringe fitted with a blunt ended 5 inch luer lock needle (Alfred Cox Ltd., Surrey, UK) was attached via straight couplings (Fisher Scientific UK).

3.6 Chemicals

Unless otherwise stated, all chemicals were of AnalaR grade and obtained from the British Drug House (BDH), Poole, Dorset, UK or Sigma Chemicals Company Ltd., Poole, Dorset, UK. All solutions were made up in glass distilled water.

3.7 Culture medium

Cultures were grown in modified Van Soest medium as described by Theodorou and Brooks (1990). The medium was prepared, at room temperature, by dissolving the following chemicals (in the order shown) in 1 l of distilled water: 0.2 g Trypticase peptone (Becton Dickinson Microbiology Systems, BBL, Cockeysville, Maryland, U.S.A.), 0.2 ml micromineral solution, 400 ml buffer, 400 ml macromineral solution and 2 ml of resazurin (Difco Ltd., East Molesley, Surrey, UK). The composition of these solutions are shown in Table 3.1. Each component was allowed to dissolve before the next component was added. The medium was mixed by the use of a magnetic stirrer and bar and gassed with CO_2 until the medium turned pink in colour (3 - 5 h). The micromineral and resazurin solutions were prepared in advance and stored in the dark at 4°C until required. All other solutions were freshly prepared before use.

The medium was dispensed into serum bottles (Phase Separations Ltd., Clwyd, UK: nominally of approximately 160 ml capacity but retailed as 125 ml bottles) containing between 0.25 - 1.00 g of substrate (details of the substrate type and weight can be found in the materials and methods sections of the relevant chapters). Bottles containing no substrate were included as controls. Medium (85 ml) was dispensed into the serum bottle using either an automated dispenser (Accuramatic 5; Accuramatic, Watlington, King's Lynn, UK) or by the use of a syringe, the syringe was gassed with CO_2 before being used to transfer the medium. Once dispensed, the medium was gassed in the bottle with CO_2 for 4 min. To complete the medium, 4 ml of freshly prepared reducing agent (0.625 g cysteine hydrochloric acid, 4 ml of 1 mol l^{-1} sodium hydroxide and 0.625 g sodium sulphide dissolved in 100 ml of distilled water, in a fume cupboard under nitrogen gas) was added to each bottle and the

Table 3.1 Composition of the solutions used in the preparation of the culture medium.

Solution	Chemical	Chemical Formula	g l^{-1} distilled water
Buffer	Ammonium hydrogen carbonate	NH_4HCO_3	4.00
	Sodium hydrogen carbonate	$NaHCO_3$	35.00
Macromineral	Di-sodium hydrogen orthophosphate dodeca-hydrate	$Na_2HPO_4.12H_2O$	9.45
	Potassium di-hydrogen orthophosphate (anhydrous)	KH_2PO_4	6.20
	Magnesium sulphate hepta-hydrate	$MgSO_4.7H_2O$	0.60
			g 100 ml^{-1} distilled water
Micromineral	Calcium chloride di-hydrate	$CaCl_2.2H_2O$	13.20
	Manganese chloride tetra-hydrate	$MnCl_2.4H_2O$	10.00
	Cobalt chloride hexa-hydrate	$CoCl_2.6H_2O$	1.00
	Ferric chloride hexa-hydrate	$FeCl_3.6H_2O$	8.00
Resazurin	Resazurin (redox indicator)		0.10

bottles sealed immediately with a butyl rubber stopper (Bellco Glass Inc.; Scientific Laboratory Supplies, Nottingham, UK). The rubber stoppers were kept in place by the use of aluminium crimp seals (Phase Separations Ltd., Clwyd, UK) fixed using a crimping tool (Phase Seperations Ltd., Queensbury, Clwyd, UK).

Once the bottles had been sealed they were chilled to 4 °C in a timer - programmable refrigerated incubator, set to heat the bottles to 39 °C several hours before the start of the experiment or they were left in an incubator at room temperature set to heat the bottles to 39 °C several hours prior to inoculation.

3.8 Inoculation

Before inoculation the pressure in the head - space of culture bottles was set to ambient pressure (zero reading on the LED [light emitting diode] digital readout meter) by inserting a needle (23 gauge x 1 inch; Fisher Scientific UK, Loughborough, UK) through the butyl rubber stopper. The needle was attached to the pressure transducer via a 3 way valve (Connecta; Scientific Laboratory Supplies, Nottingham, UK) with a 20 ml syringe on the 3rd arm. The gas was removed from the headspace of the bottle by withdrawing the syringe plunger until the LED digital voltmeter (Bailey & Machey Ltd., Birmingham, UK) displayed zero. The transducer assembly was then removed, the gas in the syringe discarded into a fume cupboard, and the whole procedure repeated on the remaining bottles. Approximately 5 minutes were required to 'zero' ten bottles. Each serum bottle was then inoculated with 10 ml of microbial inoculum using a 10 ml syringe fitted with a 21 gauge x 1.5 inch needle (Fisher Scientific UK, Loughborough, UK) Approximately 5 minutes were required to inoculate ten bottles.

After inoculation the head - space gas pressure was adjusted to ambient pressure, again using the transducer as described above.

Inoculation was carried out in a constant temperature room (39 °C) or bottles were removed, ten at a time, from an incubator, at 39 °C, to a fume cupboard, inoculated, and returned to the incubator prior to removal of the next ten bottles.
At this stage, the serum bottles contained substrate (except for control bottles or 'blanks'), 85 ml of medium, 4 ml of reducing agent and 10 ml of inoculum.

3.9 Gas accumulation measurements

Gas production was measured throughout the incubation using the manual pressure transducer technique (PTT) of Theodorou *et al.* (1994). The PTT allows both the pressure and volume of gas produced in the head-space of serum bottles to be recorded.

A three way valve was attached to the pressure transducer, with a plastic syringe attached to the side arm of the valve and a 23 gauge x 1 inch syringe needle attached to the remaining arm (Plate 2; page 20). The tap on the three way valve was adjusted so that gas could flow between the pressure transducer, the bottle and the syringe. The syringe needle was then inserted through the butyl rubber stopper into the head-space of the bottle. After the pressure in the head-space had been recorded from the digital display, the gas was withdrawn from the head-space using the syringe, until the digital display on the voltmeter displayed zero (ambient pressure). The volume of gas in the syringe was noted at this point. The pressure transducer assembly was then removed from the bottle, and the gas which had been collected in the syringe was discarded into a fume cupboard. The size of the syringe used varied depending on how much gas was produced (indicated by the pressure reading on the digital display unit) with 10, 20 and 60 ml syringes being required. The voltmeter was calibrated to read pressure as pounds per square inch (psi) in the range 0 - 15 psi with an accuracy of 0.1 psi ± 2 % (at 25 °C).

Gas accumulation was recorded frequently at the start of the incubation (after inoculation) when gas production was most rapid, with measurements becoming less frequent towards the end of the incubation. Typical reading times were at 3, 6, 9, 12,

15, 18, 21, 24, 28, 32, 37, 46, 56, 72, 96 and 140 h after inoculation. After each reading, the bottles were shaken by hand for approximately 3 seconds. Readings were carried out either in the constant temperature room (39 °C), or 15 - 20 bottles were removed from the incubator, read and returned to the incubator before removing the next 15 - 20 bottles for reading. The readings were carried out as quickly as possible to avoid the bottles cooling to below 39 °C. Every bottle took approximately 20 seconds to read, unless otherwise stated.

3.10 Analysis following incubation

After the last gas reading, the pH of the culture medium in the bottles was recorded using a pH meter (Mettler Delta 320; Fisher Scientific UK, Loughborough, UK) and liquid samples were taken for volatile fatty acid (VFA) analysis (see section 3.12.1). The cultures were then stored at 4 °C to arrest the fermentation until the contents were filtered, washed and subjected to dry matter (DM) determination.

3.10.1 Determination of residual dry matter

The bottle contents were filtered through pre-weighed sintered glass funnels (porosity 1; Gallenkamp; Fisher Scientific UK, Loughborough, UK) under reduced pressure in order to separate the supernatant from the residual particulate substrate and adherent microbial biomass. The residue was washed with two volumes (20 ml) of distilled water and freeze dried or oven dried at 60 °C to constant weight for the determination of residual DM. Given that the feed residues included microbial biomass adhering to the residue, the calculation of initial minus residual DM is apparent DM loss and is referred to as DM loss throughout this thesis.

3.11 Statistics and determination of kinetic parameters

Quattro - Pro (version 2.1 ; Borland Int. Inc.), a PC compatible spreadsheet programme, was used for processing the gas production data. The gas volume measurements for each bottle were corrected for pressure using linear regression. The corrected gas volumes were then accumulated to give the cumulative gas production profile for the incubation. The mean cumulative gas production profile for the

'blanks' (bottles containing inoculated medium but no substrate) was then subtracted from each bottle to correct for gas production from the microbial inocula.
The maximum likelihood programme (MLP; Ross, 1987) was used to fit curves to the corrected gas profiles using the model of France *et al.* (1993):

$$y = A - BQ^{t}Z^{\sqrt{t}} \qquad \text{(equation 3.1)}$$

where $Q = e^{-b}$, $Z = e^{-c}$ and $B = Ae^{bLT + c\sqrt{t}}$, y denotes cumulative gas production (ml) at time t, t is the incubation time (h), A is the asymptotic value for gas pool size (ml), L_T is the lag time (h) and b (h^{-1}) and c ($h^{-0.5}$) are rate constants.

The fitted mean gas production profiles for each treatment were analysed for significant differences using the parallel curve analysis function of MLP (Ross, 1987). This indicates whether there are significant differences between both the resultant gas pools, A and B, and the rates of gas production, b and c.

Values for DM loss, VFA production, lactic acid production and pH were analysed using the multivariate analysis of variance function of Genstat 5 (Lawes Agricultural Trust, 1993). Comparison between treatments was made by calculating the least significant difference (LSD) using the standard error of the difference (s.e.d; supplied by Genstat) and the t value at the appropriate degrees of freedom (Lindley & Scott, 1990).

3.12 Analytical Procedures

3.12.1 Volatile fatty acid analysis (VFA)

Crude culture fluid was analysed for the following VFA using gas chromatography; acetate (Ac), propionate (Pr), iso - and n - butyrate (Bu) and iso - and n - valerate (Val).

3.12.1.1 Preparation of samples

Crude culture fluid (1.2 ml) were pipetted into 1.5 ml micro-centrifuge tubes and acidified to pH 1-2, with two drops of orthophosphoric acid (H_3PO_4). Samples were stored overnight at 4 °C to allow proteinaceous material to precipitate and then centrifuged for 2 min at 1500 gs. Supernatant (1 ml) was transferred into a 2 ml glass crimp vial (Vials Direct Ltd., Macclesfield, UK) and 0.2 ml internal standard (section 3.12.1.2) added. Vials were then crimped and stored at 4 °C until analysed.

3.12.1.2 Internal standard

The internal standard consisted of 0.18 g of 2 - methyl valeric acid ($C_6H_{11}CO_2H$; Aldrich Chemical Company Ltd., Gillingham, Dorset, UK) in 100 ml distilled water plus 760 µl of concentrated orthophosphoric acid.

3.12.1.3 External standard

The external standard was composed of 15 mmol l^{-1} acetic acid, 5 mmol l^{-1} propionic acid, 0.2 mmol l^{-1} iso-butyric acid, 2 mmol l^{-1} n-butyric acid, 0.2 mmol l^{-1} iso-valeric acid and 2 mmol l^{-1} n-valeric acid in 150 mmol l^{-1} orthophosphoric acid.

External standard (1.0 ml) plus 0.2 ml of internal standard were transferred into a 2 ml glass vial, crimped and stored at 4 °C.

3.12.1.4 Gas chromatography for VFA analysis

A Chrompack 9000 gas chromatograph fitted with an automatic liquid sampler (Chrompack 911; Chrompack UK Ltd., London, UK) was used for VFA identification and quantification. The chromatograph (with split injection and flame ionisation detector) was connected to an IBM personal system/2 model 35 SX computer loaded with MOSAIC software, which facilitates data integration, manipulation and retrieval. Sample (0.5 µl) was injected into the fused silica capillary column (25 m x 32 mm internal diameter) under the following chromatographic conditions. Injector and detector temperatures were 240 °C and 260 °C respectively. The column was run at a temperature of 115 °C for 8 min, then the

temperature was increased to 200 °C, at a rate of 30 °C min^{-1}, and held at this temperature for 10 min. Helium was used as the carrier gas and arrived at the head of the column at a pressure of 75 kPa and a split flow of 200 ml min^{-1}.

3.12.2 Lactic acid analysis

Crude culture medium was analysed for L- and D- lactic acid using a Boehringer Mannheim test kit. (D - lactic acid / L - lactic acid (D - lactate / L - lactate) test kit, no.139084; Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., Lewes, East Sussex, UK).

3.12.2.1 Sample preparation and spectrophotometry

Crude culture medium (1 ml) was transferred to 1 channel in a 9 channel Eppendorf block. The samples were stored at -4 °C until analysis (approx. 48 h). Immediately before analysis, samples were centrifuged at 2500 gs for 5 min. A 10 µl aliquot of supernatant was removed from each channel and transferred into a clean cuvette block together with 100 µl of distilled water and 200 µl of buffer reagent (described below), the contents were mixed before being incubated at 37 °C for 5 min.

After incubation the absorbance of the solution was recorded at a wavelength of 340 nm and at a temperature of 20 - 25 °C using an FP 901 chemical analyser (Labsystems, Life Sciences Int. Ltd., Basingstoke, Hampshire, UK). This provided a blank reading for the absorbance measurements. L- lactate dehydrogenase solution (100 µl; L-LDH) was then added to each cuvette, mixed and incubated at 37 °C for 25 min. The absorbance of the solution was read again, followed by the addition of 100 µl of D-LDH to each cuvette. The samples then underwent a final incubation of 25 min at 37 °C before the absorbance reading was repeated to determine the total lactate content. The absorbance of the standards was measured before and after the samples to check for drift.

The principle of this technique is that L - lactic acid (L - lactate) is oxidised to pyruvate by nicotinamide - adenine dinucleotide (NAD) in the presence of L - lactate dehydrogenase (L - LDH):

$$\text{L - lactate} + NAD^{+} \xrightarrow{\text{L - LDH}} \text{pyruvate} + NADH + H^{+} \qquad \text{(equation 3.2)}$$

The equilibrium is established in favour of pyruvate formation by L - glutamate and the enzyme glutamate - pyruvate transaminase (GPT), which are present in the buffer solution (section 3.12.2.2). Their presence converts pyruvate to L - alanine and 2 - oxoglutarate:

$$\text{Pyruvate} + \text{L-glutamate} \xrightarrow{\text{GPT}} \text{L - alanine} + \text{2 - oxoglutarate} \qquad \text{(equation 3.3)}$$

The amount of NADH formed in the above reaction is relative to the amount of L - lactic acid. The increase in NADH is determined using its absorbance at 340 nm. The amount of D - lactate present is then determined by the addition of D - lactate dehydrogenase (D - LDH) which will give an absorbance reading for the NADH produced from the conversion of D - lactate.

3.12.2.2 Buffer reagent

The buffer reagent consisted of the following solutions (Boehringer Mannheim; D - lactic acid / L - lactic acid test kit);

1. glycylglycine buffer (15 ml; pH 10.0) containing 220 mg of L - glutamic acid
2. nicotinamide - adenine dinucleotide (105 mg; NAD) dissolved in 6 ml of distilled water
3. glutamate - pyruvate transaminase suspension (0.35 ml)

3.12.2.3 Standards

The standards consisted of a 50:50 mixture of L- and D- lactate at the following concentrations; 0, 100, 200, 400, 600, 800, and 1000 μg ml^{-1} (Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., Lewes, East Sussex, UK).

3.12.2.4 Quantification of lactic acid

The blank absorbance value was subtracted from both the absorbance reading for L-lactate and that for the total lactate (L- + D-lactate). The absorbance of the standards was used to create standard curves for both L- and total lactate (see appendix 2). The lactate content of the samples was then determined from the respective standard curve.

3.12.3 Non starch polysaccharide (NSP) and uronic acid determination

The NSP content of feedstuffs was determined using a modified version of the method described by Englyst and Cummings (1984), whereby alditol acetate derivatives of carbohydrate monomers derived from acid hydrosylates of washed, polymeric, de-starched samples were quantified by gas chromatography.

3.12.3.1 De-starching of the feed sample

The feed sample (150 mg) was placed into a 50 ml screw top glass centrifuge tube with a small magnetic stirrer bar (approximately 10 mm long x 3 mm wide). Dimethyl sulphoxide (2 ml; DMSO) was added to the tube and mixed for 2 min at room temperature using the magnetic stirrer. The samples were then boiled for 1 h. After boiling 8 ml of acetate buffer, warmed to 50 °C, was added to the tubes. The sodium acetate buffer (0.1 mol l^{-1}, pH 5.2) was prepared by dissolving 13.6 g sodium acetate trihydride ($CH_3OONa.3H_2O$) in 950 ml of distilled water. The pH was adjusted to 5.2 by the addition of 0.1 mol l^{-1} acetic acid. 4 ml of 1 mol l^{-1} calcium chloride (CaCl) was then added and the buffer made up to a volume of 1 l with distilled water.

Following the addition of the sodium acetate buffer the samples were cooled to between 30 and 40 °C. α - Amylase solution (0.5 ml) was then added to each tube, the contents mixed, and incubated at 42 °C for 16 - 18 h. After the first hour of the incubation the samples were mixed once more.

The α - amylase solution was prepared by mixing the contents of two Pancrex V capsules (approximately 9000 BP units α - amylase per capsule, Paines & Byrne Ltd., Greenford, UK) with 9 ml of distilled water. The mixture was then centrifuged at 1500 gs for 10 min and the resulting supernatant used as the α - amylase solution.

Following the incubation, 40 ml of absolute ethanol (CH_3CH_2OH) were added to each tube, mixed and left for 1 h at room temperature. (The ethanol is required to precipitate out soluble NSP to give an end measurement for total NSP [soluble + insoluble], where insoluble NSP only was measured the ethanol was replaced with 20 ml of sodium acetate buffer.) The samples were then centrifuged at 1500 gs for 10 min. After centrifugation the supernatant was discarded and the residue washed twice more with 50 ml of 85 % aqueous ethanol. The washing procedure involved mixing the residue with the ethanol, centrifuging at 1500 gs for 10 min and discarding the supernatant. A final wash was carried out using 40 ml of acetone (C_3H_6O) and the remaining residue dried at 65 °C, by placing the tubes on a hot plate stirrer (65 °C) until all the acetone had evaporated.

3.12.3.2 Acid hydrolysis of the residues

The dried residue remaining after enzymatic digestion, was dispersed in 2 ml of 12 mol l^{-1} sulphuric acid and mixed. The tubes containing the samples were then left at 35 °C for 1 - 1.25 h with occasional mixing to disperse the residue. Distilled water (22 ml) were added to each tube and placed in boiling water for 2 - 2.25 h, the samples were stirred continuously throughout by the use of the small magnetic stirrer in each tube. After boiling, the samples were cooled rapidly by submerging the tubes in cold water.

3.12.3.3 Preparation of alditol acetate derivatives

Internal standard (5 ml), consisting of 1 mg β-D-allose (Sigma) dissolved in 50 % saturated benzoic acid solution (v/v), was added to the acid hydrosylate (section 3.12.3.2) and mixed. One ml of this mixture was transferred into a separate test-tube and 0.2 ml of 12 mol l^{-1} ammonium hydroxide (Sigma) was added to raise the pH above 7.0. The alkalinity of the sample was tested with pH paper (Gallenkamp; Fisher Scientific UK) and if necessary, additional ammonium hydroxide was added.

Freshly prepared ammonium hydroxide / sodium borohydride ($NaBH_4$) solution (0.1 ml; 3 mol l^{-1} ammonium hydroxide containing 50 mg sodium borohydride ml^{-1}) was then added, followed by 5 µl of octan-2-ol (Sigma) to prevent foaming. The contents of the test tube were mixed thoroughly before being incubated at 40 °C for 1 h. After the incubation, 0.8 ml of glacial acetic acid (CH_3COOH) was added and the solution mixed. The acidity of the samples was then tested using pH paper and if the samples were not acidic more glacial acetic acid (in 1 µl aliquots) was added.

Each sample (0.5 ml) was transferred to a separate test tube together with 0.5 ml of 1-methylmidazole ($C_4H_6N_2$) and 5 ml of acetic anhydride ($C_4H_6O_3$). The solution was mixed and left at room temperature for 10 min before 0.9 ml of ethanol was added and mixed thoroughly. Five min after the addition of ethanol, 5 ml of distilled water were added and the solution mixed again before being left for a further 5 min.

The tubes were then placed in a cold water bath and 5 ml of 7.5 mol l^{-1} potassium hydroxide (KOH) added. After 5 min, a further 5 ml of 7.5 mol l^{-1} KOH was added. The KOH was added using an automatic dispenser (Oxford model 5200; Fisher Scientific UK, Loughborough, UK), the force of this addition was enough to mix the sample, and no further mixing was required. The tubes were left in an ice bath until the organic and aqueous phases separated (approximately 10 min).

The top, organic phase (0.2 ml) was carefully removed using a Gilson pipette and dispensed into a gas chromatography vial with sleeve inserts (Vials Direct Ltd.,

Macclesfield, UK). The vials were capped immediately after pipetting, ready for gas liquid chromatography, and in this state, (provided the seal on the vial remains airtight) the samples were stable at 5 °C indefinitely.

3.12.3.4 External standards for gas liquid chromatography

The external standard for gas chromatography consisted of 0.25 ml calibration mixture, 0.25 ml internal standard (1 mg ml^{-1} β-D-allose in 50 % saturated benzoic acid solution (v/v)) and 0.5 ml 2 mol l^{-1} sulphuric acid (H_2SO_4). The external standards were neutralised, reduced and derivatised as described for the experimental samples.

The calibration mixture was prepared using 1 mg ml^{-1} of L-rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose dissolved in 50 % saturated benzoic acid solution.

3.12.3.5 Gas-liquid chromatography for NSP analysis

A Varian 3400 chromatograph fitted with an automatic sampler (Varian 8000) and a flame ionisation detector, linked to a Dell PC with Dionex A1-450 integration software, was used for NSP identification and quantification.

The column used was glass (2.1 m x 2 mm internal diameter) packed with Supelco (100/120 mesh) coated with 3 % SP 2330. The conditions for chromatography were as follows; both the injector and detector were at a temperature of 250 °C, the column temperature was 225 °C. Nitrogen was used as the carrier gas at a pressure of 30 - 35 psi. The column was injected with 2 µl of sample.

3.12.3.6 Uronic acid determination

The uronic acid content of the hydrolysates was determined by the colorimetric method of Scott (1979). Supernatant (0.3 ml) from the acid hydrolysis stage (section 3.12.3.2) was mixed with 0.3 ml of sodium chloride / boric acid (H_3BO_3) mixture (prepared by dissolving 2 g of sodium chloride and 3 g of boric acid in 100 ml of

distilled water) in 50 ml screw-top, glass tubes. 5 ml of concentrated sulphuric acid was added to the mixture which was mixed before being incubated at 70 °C for 40 min. After the incubation, samples were cooled by placing the tubes in water. Aliquots (0.2 ml) of dimethylphenol solution (0.1 g of 3,5 dimethylphenol ($[CH_3]_2C_6H_3OH$) dissolved in 100 ml of glacial acetic acid), were added, mixed and the tubes left for 15 min. The absorbance of the samples was then measured at both 400 and 450 nm using a spectrophotometer (SP8-100 ultraviolet spectrophotometer; Pye Unicam; Fisher Scientific UK, Loughborough, UK). As the colour formation is not stable, two sets of standards were used. One set being read before, and the other set after the samples were read; the absorbance of the standards was measured at 400 and 450 nm.

The standards consisted of glucoronic acid (made up in distilled water) at the following concentrations: 25, 50, 75, 100 and 150 μg ml^{-1}. The standards were treated as described for the test samples. The mean absorbance values from the two sets of standards, with the average values from 400 nm being subtracted from the average values for 450 nm, were plotted against the uronic acid concentration to obtain a standard curve (Appendix 3). This was then used to calculate the uronic acid content of the samples using the value obtained after the absorbance at 400 nm had been subtracted from the reading at 450 nm for each sample.

3.12.4 Starch analysis

3.12.4.1 Preparation of the sample

Feed sample (150 mg) was weighed into 50 ml stoppered glass tubes and 25 ml of acetate buffer added (0.1 mol l^{-1}, pH 5.0). Thermostable α- amylase (100 μl; Termamyl; Novo Nordisk Bioindustries UK Ltd., Farnham, Surrey, UK) was added and mixed by inversion. The tubes were then placed in a boiling water bath for 60 min, cooled and 100 μl of amyloglucosidase added. The samples were then mixed before being incubated at 60 °C for 18 h. After the incubation, samples were cooled to room temperature by placing in a cold water bath. Once cool, the samples were spun at 1500 gs for 10 min. Supernatant (1 ml) was removed and diluted by a factor

of 10 with distilled water. The glucose content was then determined using the glucose oxidase method with a Baker Clinical Analyser (Baker Instruments Ltd., Egham , Surrey, UK). [The principle of this method is described in section 3.12.4.2] The sample (0.02 ml) was transferred into a clean test tube, and 2.5 ml reagent added (Encore glucose oxidase reagent set; Baker Instruments Ltd., Egham, Surrey, UK). The mixture was then vortex mixed and left, at room temperature, for 20 min. The absorbance of the samples was then read at 505 nm using the Baker Clinical Analyser (Baker Instruments Ltd., Egham, Surrey, UK). The final colour was stable for 30 min. Blanks, containing no sample but 0.02 ml of distilled water were included in the analysis, to correct for any absorbance by the reagent.

Table 3.2 Composition of the reagent used in the glucose oxidase method for the Baker Clinical Analyser (Encore Glucose Oxidase Reagent Set; Baker Instruments Ltd., Egham, Surrey, UK).

Active Ingredients	Concentration as formulated
Phenol	10.0 mmol l^{-1}
Phosphate buffer	150.0 mmol l^{-1}
4 - aminophenazone	0.4 mmol l^{-1}
Peroxidase (horseradish)	≥ 300 IU l^{-1}
Glucose oxidase (mold)	≥ 10 000 IU l^{-1}

IU = international unit; the amount of enzyme that will catalyse the transformation of 1 μmol of substrate per minute.

3.12.4.2 Principle of the glucose oxidase method

This procedure utilises the chromogenic oxygen acceptor, hydroxy benzoate - Na - 4 - aminophenazone. The sample is treated with glucose oxidase - peroxidase reagent (Encore Glucose Oxidase Reagent Set; Baker Instruments Ltd., Egham, Surrey, UK) which contains hydroxybenzoate - Na - 4 - aminophenazone. The glucose oxidase oxidises glucose to gluconic acid producing hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidises the hydroxybenzoate - Na - 4 - aminophenazone to form a stable, red quinone complex. The intensity of the colour of this complex is directly proportional to the concentration of glucose in the sample.

$$H_2O_2 + \text{hydroxybenzoate - Na - 4 - aminophenazone} \xrightarrow{\text{Peroxidase}} H_2O + \text{quinone complex}$$

(equation 3.4)

3.12.4.3 Standards

Primary aqueous glucose standards at the following concentrations (mg 100 ml^{-1}); 50, 100 and 250 (Encore Glucose Oxidase Reagent Set; Baker Instruments Ltd., Egham, Surrey, UK) were used.

3.12.4.4 Quantification of starch

Starch was quantified using the following equation:

$$\% \text{ starch} = \frac{\text{concentration of glucose (mmol } l^{-1} \text{ or mg } l^{-1}) \text{ x volume x dilution x } 0.9}{\text{sample weight (mg)}}$$

(equation 3.5)

where the concentration of glucose was obtained from the Baker Clinical analyser, the volume was 25.3 ml, the dilution was x 10 and 0.9 was the conversion factor to polysaccharides (not all the sample was converted to polysaccharide).

CHAPTER 4 - THE EFFECT OF THE PHYSICAL FACTORS, TEMPERATURE, PRESSURE AND SHAKING, ON GAS PRODUCTION

This chapter set out to investigate some of the physical factors which affect the behaviour of gases and to demonstrate their effects on gas production profiles obtained using the pressure transducer technique of Theodorou *et al.* (1994). The physical factors investigated were; temperature (section 4.1), head-space gas pressure (section 4.2) and shaking versus no shaking of the culture bottles (section 4.3).

4.1 The effect of different incubation temperatures on the production of gas from batch cultures inoculated with rumen micro-organisms

4.1.1 Introduction

As discussed in chapter 2 (section 2.2) the behaviour of a gas is affected by temperature, as described by Charles' Law. According to this law, the volume of a given amount of gas at constant pressure is proportional to the temperature, therefore the volume occupied by a gas will increase as the temperature increases. The rate of a chemical reaction is also affected by temperature as described by the Arrhenius equation and exemplified by the temperature coefficient (Q_{10}) (both are described in detail in the discussion section 4.1.4). Chemical reactions which occur in living organisms tend to have a Q_{10} of two; that is, the rate of the reaction will double for every 10 ° rise in temperature (Mandelstam & McQuillen, 1973). Temperature will therefore have a profound effect on gas production studies; with more gas being produced at higher incubation temperatures (due to the Arrhenius (Q_{10}) effect) and occupying larger volumes (as indicated by Charles' Law).

Chemical reactions in biological systems are best performed at optimal temperatures. For example, the micro-organisms of the rumen grow at an optimum temperature (39 °C; just above body temperature) at which they are most efficient (Johnson, 1966; Lowe *et al.*, 1987; Stewart *et al*, 1997). There will therefore be a limit to the increase in gas production observed as the temperature is increased above an optimal,

threshold limit. The aim of this experiment was to determine gas production from perennial ryegrass hay (*Lolium perenne*) inoculated with rumen micro-organisms and incubated at 25, 30, 39 or 45 °C. The effect of correcting all gas production profiles to s.t.p and the Q_{10} of the reaction were also investigated. This experiment was chosen because the effects on gas volume and production were anticipated to be large and the data would therefore serve as a 'bench-mark' for subsequent investigations.

4.1.2 Materials and Methods

Four identical series of bottles were used to investigate the effect of incubation temperature on the production of gas from perennial ryegrass hay (*Lolium perenne*) inoculated with buffered rumen fluid. The chemical composition of the hay is shown in Appendix 1. Each bottle contained 0.75 g DM hay, 85 ml culture medium plus 4 ml reducing agent, and was inoculated with 10 ml of microbial inoculum prepared from digesta collected from a rumenally - fistulated, hay-fed sheep. Three bottles, each containing inoculated medium but no substrate were included in each of the 4 series as substrate-negative controls. Series 1, 2, 3 and 4 were incubated in water baths (Grants; Fisher Scientific UK Ltd.) at temperatures of 25 °C, 30 °C, 39 °C and 45 °C respectively. Gas production was determined in each bottle at 3, 6, 9, 12, 16, 21, 24, 28, 32, 36, 46, 56, 72, 104.5 and 144 h after inoculation. Three bottles were analysed for VFA production, pH and DM loss at time 0 h. In addition three bottles from each water bath were harvested at 6, 12, 24, 32, 56 and 144 h in order to obtain DM loss, VFA production and pH profiles (as described in sections 3.10 and 3.12.1). The total number of bottles used in the experiment was 87 (18 bottles containing substrate plus 3 controls in each series, with an additional 3 bottles included for time 0 h determinations).

The experiment was a factorial design consisting of 4 incubation temperatures, and 3 replicate bottles (4 x 3). The gas production profiles were fitted to the model of France *et al.* (1993) and compared using the parallel curve analysis function of MLP (Ross, 1987) (section 3.11). Values for the modelled gas production parameters, DM loss, VFA production and pH were analysed using analysis of variance in Genstat 5

(Lawes Agricultural Trust, 1993). Differences between treatments were calculated using the LSD (section 3.11).

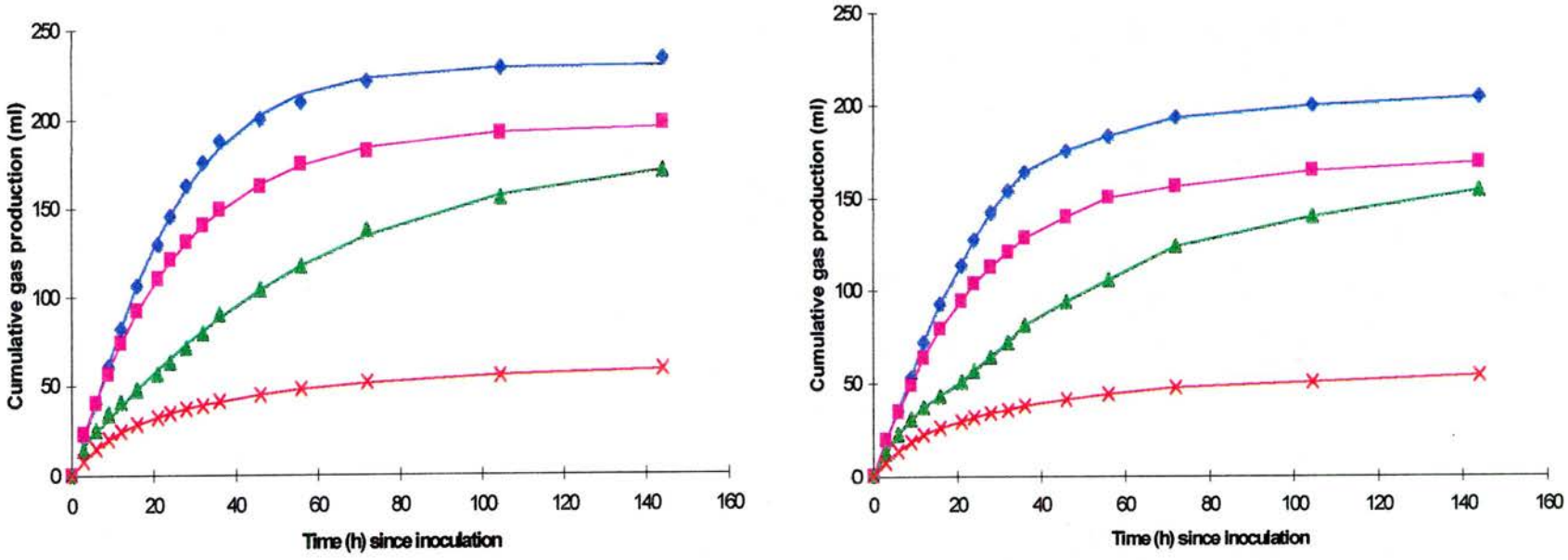
4.1.3 Results

4.1.3.1 Gas production

The gas production profiles varied with the incubation temperature (Figure 4.1.1.a). Cultures incubated at 39 °C produced the largest volumes of gas throughout the fermentation time-course, followed by cultures incubated at 45 °C, then 30 °C, with the least gas being produced at 25 °C. Correcting the gas production profiles to s.t.p values (0 °C and 1 atmosphere or 273.15 K and 101.325 kPa, respectively) resulted in lower volumes of gas at all incubation temperatures, with gas production measurements made at lower temperatures being modified to a lesser extent relative to those recorded at higher temperatures. However the overall ranking order of gas profiles was not affected (Figure 4.1.1.b). Parallel curve analysis of the fitted curves (France *et al.*, 1993) indicated significant differences in both the rate of gas production and the final volume of gas produced ($p < 0.001$; Appendix 4.1.1) between the different incubation temperatures (for both the s.t.p uncorrected and s.t.p corrected data sets). The fitted parameters and derived quantities for the gas production profiles at s.t.p are shown in Table 4.1.1. It is important to note that atmospheric pressure was not recorded during the experiment. However as atmospheric pressure in the UK ranges from 940 mb (0.93 atmospheres) to 1050 mb (1.04 atmospheres) with an average value of 1013 mb (1 atmosphere) (Hugh Quigley, Met. Office, Bracknell - personal communication) it was assumed that the experiment (conducted at sea level at Aberystwyth) was carried out at standard pressure. Analysis of variance indicated significant differences in all gas production parameters between the different incubation temperatures; these differences are detailed in Table 4.1.1. The rates of gas production, b and c, ranged from 0.0053 h^{-1} (during incubation at 25 °C) to 0.0688 h^{-1} (during incubation at 39 °C) and -0.1387 $h^{-0.5}$ (at 39 °C) to 0.1788 $h^{-0.5}$ (at 25 °C), respectively. The mean value for b for all incubation temperatures was 0.0326 ± 0.01354 h^{-1} whilst that for c was 0.0092 ± 0.06534 $h^{-0.5}$. Incubation at 25 °C produced the least gas (53.3 ml) whilst incubation

Figure 4.1.1 (**a**) Cumulative gas production profiles for perennial ryegrass (*Lolium perenne*) hay incubated with a rumen microbial inoculum at 25 (-X-), 30 (-▲-), 39 (-◆-) or 45 °C (-■-) and (**b**) cumulative gas production profiles, corrected to standard temperature and pressure (stp).

(**a**) (**b**)



(**a**) Each value represents the mean of three bottles, whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure. Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum.

(**b**) when the gas volumes were corrected to standard temperature and pressure a Q_{10} of 2 was observed between 30 and 39 °C for 9 - 56 h incubation, whilst the rate of reaction was 3 times greater at 45 °C compared with 25 °C (see section 4.1.4).

at 39 °C produced most gas (204.0 ml). The mean total gas production for all incubation temperatures was 145.0 ± 32.31 ml. The lag time, L_T (h), encountered prior to active fermentation varied with the different incubation temperatures, ranging from 0.13 h (at 30°C) to 1.12 h (at 25 °C), with a mean value across all incubation temperatures of 0.65 ± 0.244 h. The time taken to produce 50 or 95 % of the total gas production (t_{50} and t_{95}, respectively) was shortest during incubation at 39 °C (16.2 and 56.7 h for t_{50} and t_{95}, respectively) whilst incubation at 30 °C produced the longest t_{50} (26.5 h) and incubation at 25 °C the longest value for t_{95} (169.7 h). The mean values for t_{50} and t_{95} were 20.2 ± 2.22 h and 110.2 ± 25.31 h, respectively. The large value obtained for t_{50} during incubation at 30 °C is likely to be a result of the lack of a plateaux in the gas production profile (Figure 4.1.1). Incubation at 25 °C also failed to produce a plateaux for gas production, however as the rate of gas production is much slower during incubation at 25 °C compared to 30 °C the value for t_{50} has not been influenced in the same way. Similarly, t_{95} for both 25 and 30 °C was influenced by the lack of a plateaux.

Table 4.1.1 The gas production parameters (at s.t.p); rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for 0.75 g DM of perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum at 25, 30, 39 or 45 °C.

Gas production parameters	Temperature (°C)				sed	sig.
	25	30	39	45		
b (h^{-1})	0.0053^a	0.0209^b	0.0688^c	0.0355^d	0.0013	***
c ($h^{-0.5}$)	0.1788^a	-0.0150^b	-0.1387^c	0.0118^b	0.0050	***
A (ml)	53.3^a	153.9^b	204.0^c	168.7^b	6.61	**
B	66.7^a	142.9^b	174.7^c	172.5^c	6.25	**
L_T (h)	1.12^a	0.13^b	1.02^a	0.34^c	0.048	**
t50 (h)	19.40^a	26.54^b	16.20^a	18.66^a	1.381	**
t95 (h)	169.7^a	132.3^b	56.7^c	82.0^c	12.30	*

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles corrected to s.t.p. shown in Figure 4.1.1. Values in rows not bearing the same superscripts differ significantly (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The analysis of variance tables for the above data are shown in Appendix 4.1.2 - 4.1.8.

4.1.3.2 Dry Matter Loss

DM loss at the different incubation temperatures followed a similar pattern to the corresponding gas production profiles (Figure 4.1.2). DM loss was similar during the early stages of incubation for all incubation temperatures, becoming significantly different as the incubation proceeded ($p < 0.05$; Table 4.1.2). Significantly more DM was lost with time as the incubation proceeded at 30, 39 and 45 °C ($p < 0.05$). The differences between DM loss at the start and end of the incubation was greatest during incubation at 39 °C (DM loss differed by 469.3 mg g^{-1} between 6 and 144 h incubation), followed by incubation at 45 and 30 °C (differences of 413.2 and 400.8 mg g^{-1}, respectively), whilst there was no significant difference in DM loss over time during incubation at 25 °C (a difference of only 16.0 mg g^{-1} between incubation at 6 and 144 h incubation). Throughout the incubation DM loss was greatest in the bottles

Figure 4.1.2 DM loss during incubation of perennial ryegrass (*Lolium perenne*) hay with a rumen microbial inoculum at 25 (-X-), 30(-▲-), 39 (-◆-) or 45 °C (-■-).

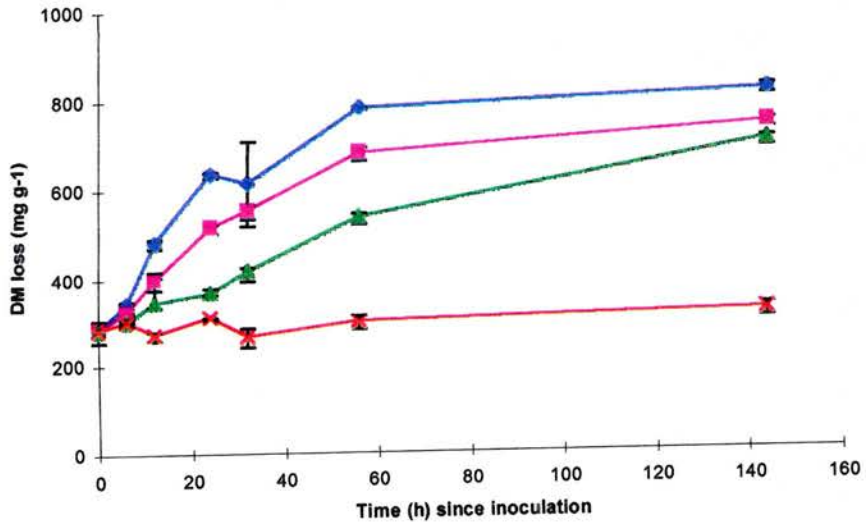
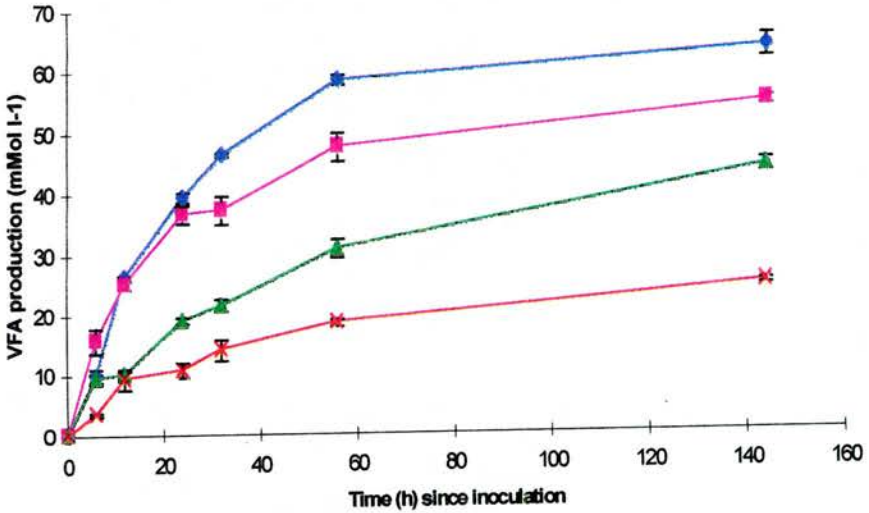


Figure 4.1.3 Total volatile fatty acid (VFA) production during incubation of perennial ryegrass (*Lolium perenne*) hay with a rumen microbial inoculum at 25 (-X-), 30 (-▲-), 39 (-◆-) or 45 °C (-■-).



Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum. Each value is the mean value of three bottles. Three bottles were harvested at 6, 12, 24, 32, 56 and 144 h after inoculation for DM loss and VFA production measurements.

incubated at 39 °C, followed by 45 °C, then 30 °C with least degradation occurring in the bottles incubated at 25 °C.

Table 4.1.2 Dry matter (DM) loss (mg g^{-1}) from perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum at 25, 30, 39 or 45 °C.

Incubation	Temperature (°C)				
time (h)	25	30	39	45	sed
6	$299.7^{k,l,m}$	$299.4^{k,l,m}$	$343.0^{j,k,l}$	$322.5^{k,l,m}$	
12	267.8^{m}	$344.4^{j,k,l}$	$480.1^{g,h}$	$396.8^{i,j}$	
24	$308.2^{k,l,m}$	$367.1^{i,k}$	$634.2^{d,e}$	511.4^{g}	
32	263.4^{m}	$417.4^{h,i}$	$613.1^{e,f}$	$550.0^{f,g}$	
56	$295.8^{l,m}$	535.1^{g}	$779.7^{a,b}$	$677.5^{c,e}$	
144	$315.7^{k,l,m}$	$700.2^{c,d}$	812.3^{a}	$735.7^{b,c}$	35.26

Values not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance table for the above data is shown in Appendix 4.1.9.

4.1.3.3 Volatile fatty acid production (VFA) production

Total VFA production increased throughout the fermentation with the different incubation temperatures, following a pattern similar to the gas production profiles (Figure 4.1.3). VFA production tended to be highest in the bottles incubated at 39 °C, whilst bottles incubated at 45 °C produced significantly more VFA than those incubated at 30 °C ($p < 0.05$), which in turn produced significantly more than bottles incubated at 25 °C ($p < 0.05$) (Table 4.1.3). Values for individual VFA production; acetate (Ac), propionate (Pr), butyrate (Bu) and valerate (Val) are shown as a molar percentage of the total VFA production (Table 4.1.3). In general, the molar percentage of acetate and butyrate produced during incubations was greater in bottles incubated at 39 and 45 °C compared to those incubated at 25 or 30 °C. Whilst the molar percentages of propionate and valerate were lower in bottles incubated at 39 and 45 °C, compared with bottles incubated at 25 or 30 °C. At the end of the incubation, similar VFA molar percentages were found in bottles incubated at 39 and 45 °C; 63 Ac: 24 Pr: 9 Bu: 4 Val and 65 Ac: 24 Pr: 8 Bu: 3 Val, respectively. Whilst

the VFA data for incubation at 25 and 30 °C showed lower percentages of Ac and higher percentages of Pr, 54 Ac: 33 Pr: 8 Bu: 5 Val and 57 Ac: 31 Pr: 9 Bu: 3 Val, respectively, than those found at 39 and 45 °C ($p < 0.05$).

4.1.3.4 Changes in batch culture pH

The pH of batch cultures varied throughout the incubation for the various incubation temperatures (Table 4.1.4). However, the differences were small and unlikely to be biologically significant. The range of pH observed varied by 0.5 units from 6.50 at 144 h incubation at 30 °C to 6.98 at 12 h incubation at 45 °C. The mean pH value for all incubations was 6.76 ± 0.024.

Table 4.1.4 pH of culture medium during incubation of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum at 25, 30, 39 or 45 °C.

Incubation	Temperature (°C)				
time (h)	25	30	39	45	sed
6	$6.74^{f,g,h}$	$6.79^{c,d,e,f}$	$6.75^{f,g,h}$	6.94^{a}	
12	$6.84^{b,c}$	6.86^{b}	$6.78^{d,e,f,g}$	6.98^{a}	
24	$6.81^{b,c,d,e}$	$6.82^{b,c,d}$	$6.76^{e,f,g}$	6.95^{a}	
32	$6.73^{g,h}$	$6.73^{g,h}$	$6.70^{h,i}$	$6.77^{d,e,f,g}$	
56	6.67^{i}	6.56^{j}	6.57^{j}	6.60^{j}	
144	$6.72^{g,h,i}$	6.50^{k}	$6.75^{f,g,h}$	6.85^{b}	0.02907

Values not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance table is shown in Appendix 4.1.15.

Table 4.1.3 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced from perennial ryegrass hay (*Lolium perenne*) during incubation with a rumen microbial inoculum at 25, 30, 39 and 45 °C.

VFA	Incubation	Temperature (°C)				s.e.d
	time (h)	25	30	39	45	
Total	6	3.57^{a}	9.57^{b}	$9.78^{b,c}$	$15.71^{d,e}$	
(mmol l^{-1})	12	9.27^{b}	$10.13^{b,c}$	26.46^{h}	$25.23^{g,h}$	
	24	$10.73^{b,c}$	$19.17^{e,f}$	39.50^{j}	36.67^{j}	
	32	$13.34^{c,d}$	$21.76^{f,g}$	46.47^{k}	37.28^{j}	
	56	$18.59^{e,f}$	30.96^{i}	58.55^{m}	47.52^{k}	
	144	$24.00^{g,h}$	43.88^{k}	63.48^{n}	54.41^{l}	1.828
Acetate	6	44.14^{a}	$54.66^{b,d}$	$56.89^{c,d,e,f}$	$59.59^{e,h}$	
(molar %)	12	53.02^{b}	$55.11^{b,c}$	$59.49^{e,h}$	60.24^{h}	
	24	53.76^{b}	$57.48^{c,e}$	$62.81^{g,h}$	$63.40^{g,i}$	
	32	$54.97^{b,c}$	$57.05^{c,d,e}$	$62.73^{g,h}$	$64.71^{g,i}$	
	56	53.80^{b}	$58.68^{e,h}$	$63.55^{g,i}$	65.73^{i}	
	144	$54.30^{b,f}$	$57.34^{c,d,e}$	$63.18^{g,i}$	$65.14^{g,i}$	1.338
Propionate	6	44.39^{a}	35.52^{b}	$33.37^{b,c}$	$30.59^{d,e}$	
(molar %)	12	35.84^{b}	35.06^{b}	30.08^{e}	29.92^{e}	
	24	35.35^{b}	$33.53^{b,f}$	$26.44^{g,h}$	26.79^{g}	
	32	35.34^{b}	$32.97^{b,d}$	$25.71^{g,h}$	$25.75^{g,h}$	
	56	35.10^{b}	$31.43^{c,d,e,f}$	$24.13^{g,h}$	$24.71^{g,h}$	
	144	$33.00^{b,d}$	$30.94^{c,d,e,f}$	23.93^{h}	$24.26^{g,h}$	1.324
Butyrate	6	$6.25^{j,k}$	$6.21^{j,k}$	5.72^{k}	$6.49^{h,k}$	
(molar %)	12	$6.76^{f,g,h,k}$	5.79^{k}	$7.77^{b,c,d,e,f}$	$6.59^{h,k}$	
	24	$6.30^{i,j,k}$	5.75^{k}	$8.01^{a,e}$	$7.34^{d,e,h,i}$	
	32	5.90^{k}	$6.76^{f,g,h,k}$	$8.41^{a,d}$	$7.05^{e,h,j}$	
	56	$6.62^{g,h,k}$	$7.68^{c,d,e,g}$	$8.63^{a,c}$	$7.39^{d,e,h}$	
	144	$7.78^{b,c,d,e,f}$	$8.79^{a,b}$	8.98^{a}	$7.92^{a,e}$	0.535

Valerate	6	5.28^{a}	$3.62^{f,h,i,j}$	$4.02^{c,d,f}$	$3.33^{f,g,i,j,k}$	
(molar %)	12	$4.38^{b,e,c,d}$	$4.04^{c,d,f}$	$2.66^{k,m,n}$	$3.25^{g,i,j,k}$	
	24	$4.58^{a,b,c}$	$3.13^{h,i,j,l}$	$2.74^{k,m,n}$	$2.46^{l,m,n}$	
	32	$3.79^{d,f,g,h}$	$3.02^{i,j,m}$	$3.14^{h,i,j,k,l}$	$2.48^{l,m,n}$	
	56	$4.47^{b,d}$	$2.37^{m,n}$	$3.69^{e,j}$	2.20^{n}	
	144	$4.92^{a,b}$	$2.91^{i,m,n}$	$3.91^{c,d,f,g}$	$2.66^{k,m,n}$	0.358

Values for each parameter not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables for the above data are shown in Appendix 4.1.10 - 4.1.14.

4.1.4 Discussion

Temperature had a significant effect on both the total gas production and the rate of gas production during incubation of 0.75 g perennial ryegrass hay with a rumen microbial inoculum. The effect of temperature on the volume of a gas is given by Charles' Law (section 2.2.3.2). Due to this law and also the influence of Boyle's Law (section 2.2.3.1) it is necessary to convert gas values to s.t.p in order to make comparisons.

The effect of temperature on the rate of a chemical reaction can be described using the Arrhenius equation:

$$k = Ae^{-E/RT}$$

where k is the rate constant, T is the temperature in degrees Kelvin, R is the gas constant, A is a constant and E is a second constant called the 'activation energy' of the reaction.

From this relationship, it follows that a small change in temperature causes a large change in the value of k and therefore a large change in the rate of the chemical reaction. The ratio of the rate of progress of any reaction or process, at a given temperature, to the rate at a temperature 10 ° lower is known as the temperature coefficient, or Q_{10} (The Wordsworth Dictionary of Science and Technology, 1995).

In general, the chemical reactions which occur in living organisms have a Q_{10} of approximately two. That is, the rate of the reaction will double for every 10 ° rise in temperature (Mandelstam & McQuillen, 1973). This increase in rate is due to the input of energy in the form of heat, which enables the activation energy of the reaction to be reached more quickly, hence increasing the rate of the reaction. However, if the chemical reaction takes place in a biological system and is mediated by enzymes, once a certain temperature has been reached, the relationship no longer applies as the enzymes and cofactors involved in the reaction are often heat labile and are inactivated or degraded at the higher temperatures. Therefore, the optimum temperature for growth results from a balance between the increased rate of reaction and the increased rate of thermal inactivation of the enzymes (Mandelstam & McQuillen, 1973).

The temperature of the rumen is usually between 39.0 °C and 40.5 °C. However it may drop to 37 °C after feed ingestion or rise to 41 °C during active fermentation (Hungate, 1966). For the *Neocallimastix* fungi described by Lowe *et al.* (1987), the optimum temperature for zoospore development was found to be 39 °C, although significant growth occurred between 36 and 41 °C, and no growth was observed at temperatures of 30 °C or lower. Studies by Orpin and Greenwood (1986) found the optimum temperature of the fungus, *Neocallimastix partriciarum*, to be 40 °C, although some growth also occurred at 45 °C. The bacteria of the rumen are also affected by temperature. Their optimum growth occurs between 30 and 45 °C. However growth may also occur, at a very slow rate, between 10 and 20 °C (Dehority & Grubb, 1980). The rumen bacteria are very sensitive to high temperatures (above 45 °C), and a loss in activity generally occurs when they are exposed to high temperatures for a significant period of time (Johnson, 1966). Similarly the enzymes of the rumen bacteria and fungi (for example, the cellulolytic, hemicellulolytic and amylolytic enzymes) are sensitive to temperature. As the temperature increases the rate of the enzyme-catalysed reactions also increases, however as the enzymes are proteins at temperatures above 40 - 50 °C they become denatured and the rate of the reaction decreases (Bergmeyer, 1978). In this study, the low values for DM loss

obtained during incubation at 25 °C confirm the low activity of the rumen micro-organisms at this temperature. In addition to affecting the growth of rumen bacteria, temperature may also influence their attachment to feed particles (Minato & Suto, 1978; Dehority & Grubb, 1980). The rumen bacteria, *Bacteroides succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* have been shown to attach to purified cellulose *in vitro* at 38 °C, but not at 4 °C (Minato & Suto, 1978).

In this study gas production, DM loss and VFA production all increased with increasing incubation temperature, from 25 °C, to 39 °C. Whilst, at 45 °C all measurements were lower than those at the optimum 39 °C. When the gas production profiles at the different incubation temperatures were corrected to s.t.p (Figure 4.1.1b), the rate and volume of gas produced at 39 °C was seen to be approximately double that during incubation at 30 °C between 9 and 56 h after inoculation. The difference in rates before 9 h incubation is likely to be less than double as the micro-organisms will be acclimatising to their new environments, whilst after 56 h incubation the substrate is depleted and most of the gas has been produced, hence the volume and rate of gas production, although greater at 39 °C compared to 30 °C, are less than double. At 45 °C, the rate of reaction would be expected to be four times greater than that at 25 °C, however as the optimum temperature for the reaction is 39 °C the rate of the reaction was slower at 45 °C, the rate being only three times greater at 45 °C compared to 25 °C.

DM loss was similar at the beginning of the incubation for all temperatures, and this may be explained by the solubilisation of material rather than differences in degradation due to the different incubation temperatures. In addition to solubilisation of the hay, the high DM loss value obtained at time zero (Figure 4.1.2) may be explained by the loss of fine hay particles through the filter. Throughout the incubation, DM loss tended to be greatest with the bottles incubated at 39 °C, indicating that this temperature was more suitable for substrate degradation than temperatures of 25, 30 or 45 °C. Alexander (1969) reported similar findings during incubation of forages in a modified version of the Tilley *et al.* (1960) technique; with

an overall temperature coefficient of digestibility, measured between 35.5 °C and 42 °C, of 0.67 units per °C. He also investigated the effect of continually cycling the temperature through these limits once per hour throughout the incubation period and found that the resulting digestibilities, although generally lower, were not significantly different from those values obtained during incubation at a constant temperature of either 38.5 or 42 °C.

The molar percentages of VFA produced also varied with the incubation temperature. This suggests that either (1) different micro-organisms were selectively enriched for at the different incubation temperatures (the majority of rumen micro-organisms have an optimum temperature of 39 °C (Hungate, 1966; Orpin, 1975), however some micro-organisms are more resistant to changes in temperature than others) or that (2) the micro-organisms used different pathways to ferment the substrate at the different incubation temperatures. Several different pathways are used during the fermentation of substrate by micro-organisms (Miller & Wolin, 1979; Smith & Bryant, 1979; Russell & Hespell, 1981; Russell & Wallace, 1997). The pathway chosen for substrate fermentation is known to be affected by factors such as rumen pH and substrate availability (Russell & Wallace, 1997). Therefore incubation temperature may also influence the fermentation pathway. The different molar percentages of VFA would also affect the resulting gas production profiles as the type of VFA produced influences the amount of gas which is produced (see section 5.1).

As discussed in section 2.2.3.4, values for s.t.p were adopted in order to compare gases without the influence of volume changes related to temperature and pressure (Morris, 1983; Atkins & Clugston, 1986). However use of these values has not been adopted as a standard procedure in gas production studies. Atmospheric pressure in the UK ranges from 0.93 to 1.04 atmospheres (Hugh Quigley, Met. Office, Bracknell - personal communication), therefore correction to standard pressure will have little effect on gas production measurements made in the UK. However where atmospheric pressure is greater, for example Addis Ababa, Ethiopia, atmospheric pressure will have a large influence on the resulting gas production profiles (Sileshi, 1994). In

order to compare gas production profiles across laboratories, particularly where work is conducted at different altitudes, gas production profiles should be corrected to standard pressure. As shown in this experiment correction to standard temperature did not result in similar gas production profiles for all incubation temperatures, as temperature influences volume via both physical effects (gases expand with increasing temperatures) and biological effects (Q_{10} and rates of gas production i.e. gas volume increases and production increases with increasing temperature). For this reason correction of gas production data to standard temperature (0 °C) is not recommended as this correction may give the impression that gas production experiments can be conducted at different incubation temperatures when in fact temperature affects many factors, such as microbial activity and the rate of reactions, in addition to physical volume.

In conclusion, gas production studies with rumen micro-organisms should be conducted at 39 °C as this is the optimal temperature for their growth (therefore reducing the incubation time required to produce an asymptote of gas production and making differences between feeds easier to detect). As large differences were noted in the gas production profiles between temperatures, care should be taken to ensure that the fermentation bottles (and their head-spaces) remain at 39 °C throughout the incubation. Ideally a constant temperature room should be used, however where this is not possible reliable incubators and water baths can be used. When using water baths the water level should be checked regularly to ensure the bottles remain submerged in the water to the same extent throughout the incubation. Where incubators are used it is important to remove only a few bottles at a time for gas production measurements (which should be taken as quickly as possible) to avoid bottles cooling down. In addition, where gas production studies are conducted at elevations above sea level, the volumes should be standardised to 1 atmosphere.

4.2 The effect of relieving head-space pressure every 2, 4 or 6 hours on the production of gas in batch cultures inoculated with rumen micro-organisms.

4.2.1 Introduction

During gas production studies using the manual pressure transducer technique, the pressure which builds up in the head-space of culture bottles is relieved at predetermined intervals throughout the incubation (initially every 3 h, becoming less frequent as the incubation proceeds). As the gas is accumulating in a confined volume, the pressure will increase with time (in accordance with Boyle's Law). In many chemical reactions, the accumulation of end products results in inhibition of the chemical reaction (Morris, 1983). For example, increased carbon dioxide pressure has been shown to inhibit the fermentation rate of glucose by the yeast *Saccharomyces cerevisiae* during wine making (Kunkee & Ough, 1966). Therefore it is possible that the build up of gas in the head-space of culture bottles may inhibit or alter the fermentation. Moreover, due to the effect of gas pressure on gas solubility (Henry's Law; section 2.2.7.3.1), more gas will go into solution, not appearing in the gas phase, as the pressure is increased. In recognition of these two processes, in the technique of Theodorou *et al.* (1994) the head-space pressure is relieved at intervals to avoid potential inhibition of the fermentation and to limit the amount of gas in solution. However, the extent to which these processes affect gas production studies has not been determined.

The aim of the following series of experiments was therefore to investigate the effect of relieving the head-space pressure every 2, 4 or 6 hours throughout the incubation, in order to quantify the effect of head-space gas pressure on the developing gas production profiles and to arrive at a suitable protocol for measuring gas production. Firstly, in two experiments (4.2.1 and 4.2.2), the reading frequency (2, 4 or 6 h) was considered alongside the nature of the substrate by incubating (1) a rapidly degradable substrate, naked oats, (experiment 4.2.1) and (2) a slowly degradable substrate, hay (experiment 4.2.2). In experiment 4.2.3, the reading frequency and the quantity of substrate incubated (0.25, 0.50, 0.75 and 1.00 g naked oats) were varied.

Whilst in the final experiment (4.2.4), the possibility of allowing the pressure transducer assembly to remain in the bottle head-space for 20, 60 or 120 s, to determine if increasing the duration of the reading in bottles read at different frequencies would produce any differences in the resultant profiles.

4.2.2 Materials and Methods

Three identical series of bottles were used in experiment 4.2.1 to investigate the effect of head-space gas pressure on the gas production profiles. Each bottle contained 1.00 g DM of naked oats (appendix 1), 85 ml of culture medium and 4 ml reducing agent (as described in section 3.7). Bottles were inoculated with 10 ml ovine rumen fluid (section 3.2). The bottles were then incubated at 39 °C for 40 h (series 1 and 2) or 42 h (series 3). Gas production was recorded at different reading frequencies of 2, 4 and 6 h for bottle series 1, 2 and 3 respectively. Bottles were harvested every four hours (series 1 and 2) or every six hours (series 3) for DM loss, volatile fatty acid (VFA) production and pH measurements (section 3.12.1 and 3.10). All available data have been used in DM loss and VFA production figures, whilst tables indicate the statistical analysis between reading frequencies for data values at coincident time points.

Experiments 4.2.2, 4.2.3 and 4.2.4 followed the same protocol as experiment 4.2.1 (described above) with the following exceptions; (4.2.2) a slowly degradable substrate; 0.75 g perennial ryegrass (*Lolium perenne*) hay (appendix 1) was used instead of the rapidly degraded naked oats, (4.2.3) different quantities of substrate; 0.25, 0.50, 0.75 and 1.00 g naked oats were incubated and (4.2.4) 1.00 g naked oats was incubated to investigate the effect of allowing the pressure transducer assembly to remain in the head-space of the bottle for 20, 60 or 120 s. DM loss, VFA production and pH were not measured in experiments 4.2.3 and 4.2.4. For experiment 4.2.4, the total incubation time was 12 h for all bottles.

Each of the experiments (4.2.1 - 4.2.4) was a factorial design consisting of 3 reading frequencies (2, 4 or 6 h), 3 replicate bottles and either (1) 4 coincident incubation

time points (12, 24, 36 and 40 / 42 h since inoculation; experiments 4.2.1 and 4.2.2), (2) 4 different substrate weights (0.25, 0.50, 0.75 and 1.00 g; experiment 4.2.3) or (3) 3 different dwell times (20, 60 or 120 s; experiment 4.2.4). The gas production profiles were fitted to the model of France *et al.* (1993) and compared using the parallel curve analysis function of MLP (Ross, 1987) (section 3.11). Values for the modelled gas production parameters, DM loss, VFA production and pH were analysed using analysis of variance in Genstat 5 (Lawes Agricultural Trust, 1993). Differences between treatments were calculated using the LSD (section 3.11).

4.2.3 Results

4.2.3.1 *Nature of the substrate (naked oats or hay) (Experiments 4.2.1 and 4.2.2)*

4.2.3.1.1 Gas production

Gas production profiles for 1.00 g naked oats and 0.75 g ryegrass hay are shown in Figures 4.2.1 and 4.2.2, respectively. The frequency of readings had a significant effect on the gas production profiles obtained for both naked oats and hay ($p < 0.01$; appendix 4.2.1 and 4.2.2 for naked oats and hay respectively). Series 1, where gas production was read every 2 h, showed the highest gas production whilst lesser amounts of gas were produced in series 2 and 3 (read every 4 and 6 h) respectively. Towards the end of the incubation, the gas production profiles of the 4 and 6 h bottles tended towards the 2 h profile, suggesting that if the incubation had been continued for a longer period of time, the final gas pools may have been the same for all three series of bottles. Although the gas production profiles indicated that fermentation of naked oats was complete by the end of the incubation, the incubation period was not sufficiently long for complete fermentation of hay (Figure 4.2.2). Moreover, the differences between the different reading frequencies were less pronounced during incubation of the hay compared with the naked oats (Figures 4.2.1 and 4.2.2).

Given that the hay did not reach an asymptote for gas production within the incubation period, the error associated with the parameter estimates made curve fitting unrealistic for these profiles (France *et al.*, 1993). The gas profiles from this experiment are not therefore discussed any further. Fitted parameters and derived

Figure 4.2.1 Cumulative gas production during incubation of 1.00 g naked oats (*Avena nuda*) with a rumen microbial inoculum where gas production was recorded every 2 (-◆-),4 (-■-) or 6 h (-▲-).

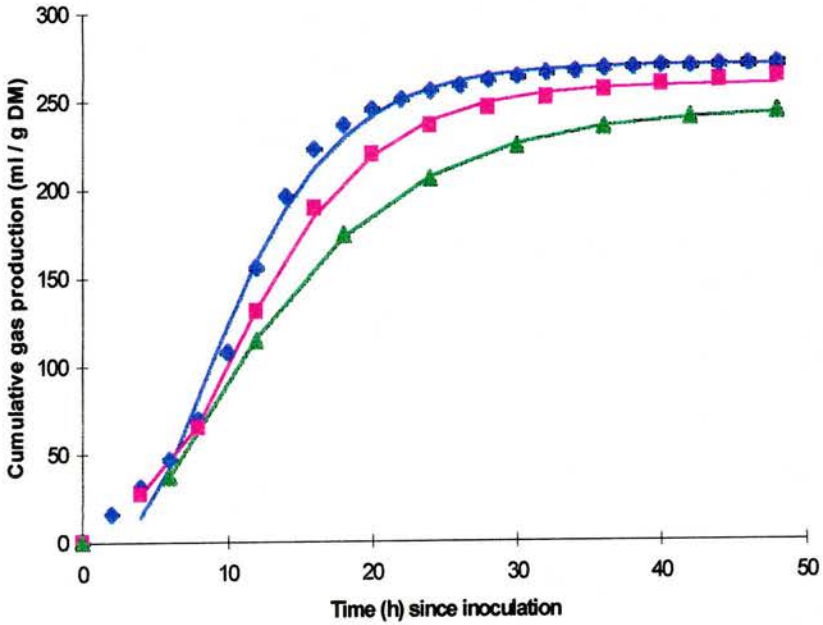
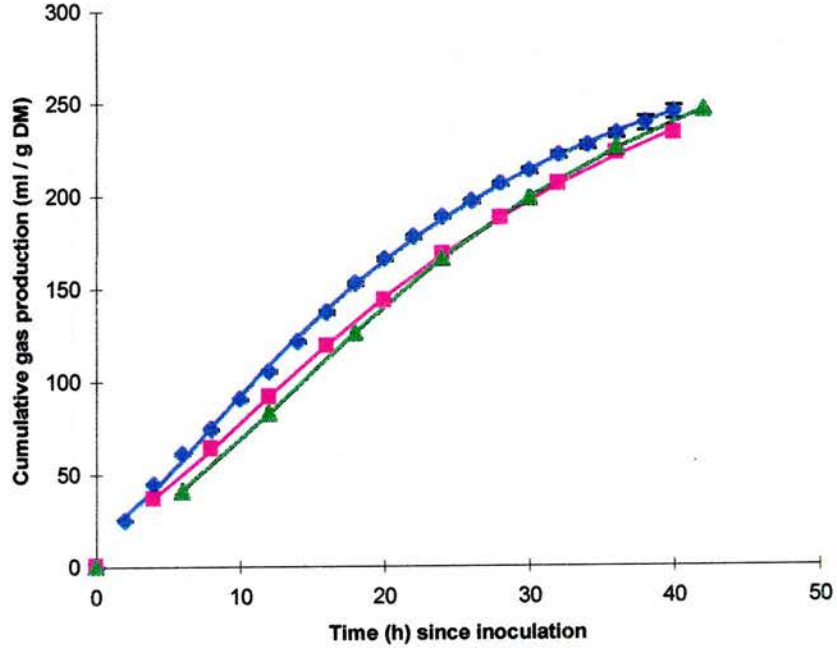


Figure 4.2.2 Cumulative gas production during incubation of 0.75 g perennial ryegrass (*Lolium perenne*) hay with a rumen microbial inoculum where gas production was recorded every 2 (-◆-), 4 (-■-) or 6 h (-▲-).



Each value represents the mean of three bottles, whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure. Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum.

quantities for the gas production profiles (France *et al.*, 1993), from naked oats, are shown in Table 4.2.1. Significant differences were seen between the gas production parameters for naked oats read every 2, 4 or 6 h, and these are detailed in Table 4.2.1. The rates of gas production, b and c, ranged from 0.1507 h^{-1} (when bottles were read every 6 h) to 0.3061 h^{-1} (for bottles read every 2h) and from -1.0960 $h^{-0.5}$ (for bottles read every 2 h) to -0.4030 $h^{-0.5}$ (for bottles read every 6h) for b and c, respectively. The mean value for b across all reading intervals was 0.2448 ± 0.04777 h^{-1} whilst that for c was -0.8630 ± 0.23001 $h^{-0.5}$. The predicted asymptote of gas production (A) was greatest when a reading frequency of 2 h was employed (268.3 ml) and lowest when the reading frequency was 6 h (246.8 ml). The mean value for A for all reading frequencies was 258.1 ± 6.23 ml. The lag time, L_T, ranged from 1.79 h (for bottles read every 6 h) to 3.85 h (for bottles read every 4 h) with a mean value for all reading frequencies of 2.95 ± 0.608 h. The shortest values for both t_{50} and t_{95} occurred in the bottles which were read every 2 h (10.46 and 23.91 h for t_{50} and t_{95}, respectively) whilst the longest values were observed in bottles which were read every 6 h (12.40 and 33.81 h for t_{50} and t_{95}, respectively). The mean values for t_{50} and t_{95} were 11.48 ± 0.562 h and 28.19 ± 2.930 h respectively.

4.2.3.1.2 Dry matter loss

DM loss for naked oats and hay at coinciding time points throughout the incubation for all reading frequencies (2, 4 and 6 h) are shown in Table 4.2.2. DM loss was not significantly different for the different reading frequencies at most incubation time points for both naked oats and hay.

Apparent DM loss from the naked oat substrate was complete within 24 h (Figure 4.2.3). However gas production, as shown in Figure 4.2.1, continued until approximately 34 h after inoculation. This may be explained by the method used to determine DM loss (filtration through sinter glass crucibles of porosity 1) and the nature of naked oats. A significant proportion of naked oats is soluble in the form of starch (see Table 5.1.1). Therefore, when the (ground) naked oats are suspended in

culture medium, a significant proportion of the ground substrate will dissolve, giving rise to the large initial decrease in apparent substrate DM at zero incubation time (Figure 4.2.3). Much of this substrate, although lost from the DM fraction, will be converted to fermentation end-products during the course of the incubation. Therefore, with naked oats, gas was produced after all the DM had been lost, as the soluble component of the naked oats was available for fermentation. In contrast when hay was treated in the same manner DM loss was seen to increase from 24 h to 40 - 42 h incubation (Table 4.2.2 and Figure 4.2.4). As hay has a much smaller soluble component fraction than naked oats, gas production was more closely correlated to DM loss.

Unlike the gas production profiles for naked oats and hay, DM loss profiles were unaffected by the different reading frequencies.

Table 4.2.1 The gas production parameters (France *et al.*, 1993); rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T) and time taken to produce 50 or 95 % of the total gas production (t_{50} and t_{95} respectively) for 1.00 g naked oats (*Avena nuda*) incubated with a rumen microbial inoculum in the pressure transducer technique, where gas production was recorded every 2, 4 or 6 h.

Gas production parameters	Reading frequency (h)			sed	Significance
	2	4	6		
b (h^{-1})	0.3061^a	0.2777^b	0.1507^c	0.0069	*
c ($h^{-0.5}$)	-1.096^a	-1.090^a	-0.403^b	0.0393	***
A (ml)	268.3^a	259.3^b	246.8^c	2.89	*
B	95.3^a	79.2^a	194.3^b	11.30	***
L_T (h)	3.20^a	3.85^b	1.79^c	0.2093	*
t50 (h)	10.46^a	11.57^b	12.40^c	0.1666	*
t95 (h)	23.91^a	26.85^b	33.81^c	0.321	***

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 4.2.1. Values in rows not bearing the same superscripts differ significantly. The analysis of variance tables for the above data are shown in Appendix 4.2.1.3 - 4.2.1.9.

Figure 4.2.3 Dry matter (DM) loss during incubation of 1.00 g naked oats (*Avena nuda*) with a rumen microbial inoculum where gas production was recorded every 2 (-◆-),4 (-■-) or 6 h (-▲-).

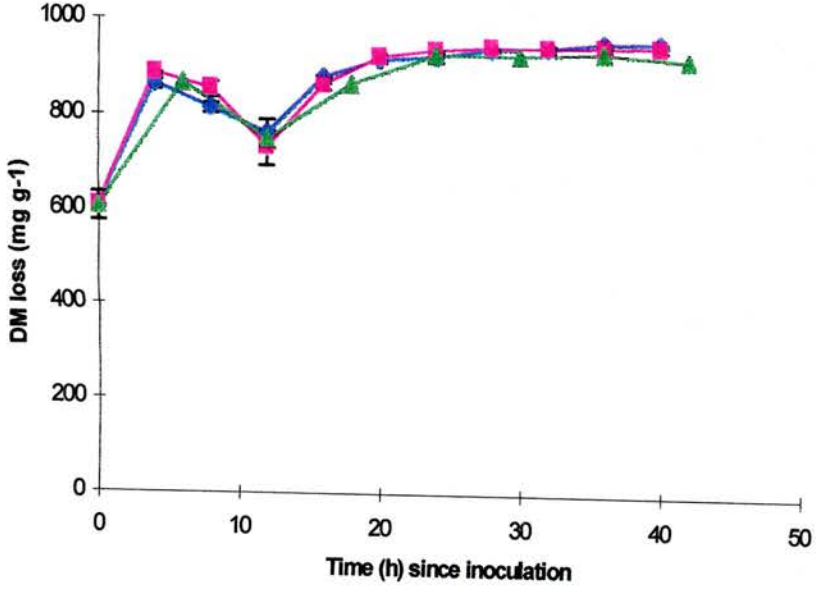
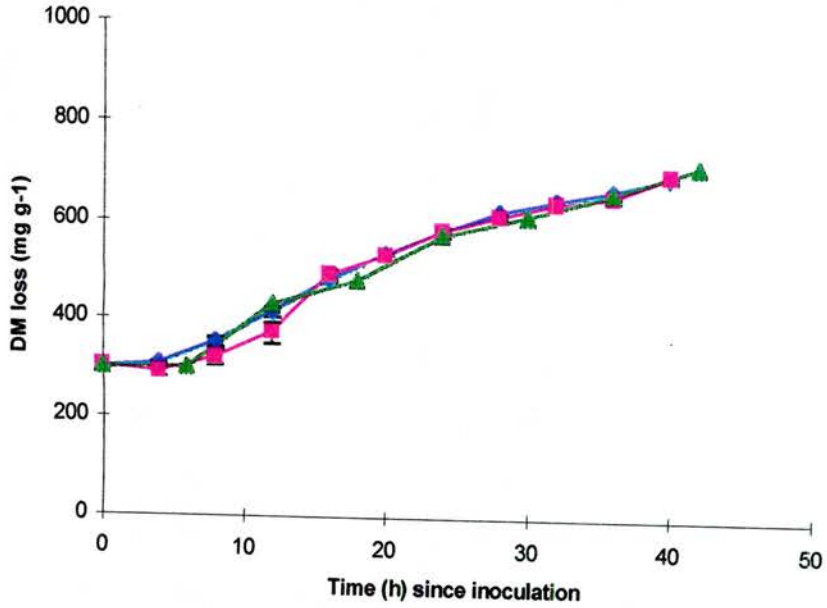


Figure 4.2.4 Dry matter (DM) loss during incubation of 0.75 g perennial ryegrass (*Lolium perenne*) hay with a rumen microbial inoculum where gas production was recorded every 2 (-◆-),4 (-■-) or 6 h (-▲-).



Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum. Each value is the mean value of three bottles. Three bottles were harvested every 4 h for DM loss measurements in bottles read every 2 and 4 h, whilst bottles read every 6 h were harvested for DM loss measurements every 6h.

Table 4.2.2 Dry matter loss (mg g^{-1}) from 1.00 g DM naked oats (*Avena nuda*) or 0.75 g DM perennial ryegrass (*Lolium perenne*) hay at 12, 24, 36 and 40 / 42 h incubation with a rumen microbial inoculum, where gas production was recorded every 2, 4 or 6 hours.

Substrate	Reading frequency (h)	Incubation time (h) 12	24	36	40 / 42*	s.e.d
Naked oats	2	840.5^{a}	935.8^{b}	898.5^{c}	$922.9^{b,c}$	
	4	826.7^{a}	937.6^{b}	937.5^{b}	$922.4^{b,c}$	
	6	821.4^{a}	$915.7^{b,c}$	902.7^{c}	$913.9^{b,c}$	11.55
Hay	2	415.4^{a}	583.7^{c}	668.6^{d}	697.5^{e}	
	4	372.7^{b}	582.9^{c}	653.9^{d}	698.3^{e}	
	6	431.0^{a}	576.6^{c}	657.6^{d}	719.1^{e}	13.65

*Bottles read every 2 or 4 h were incubated for a total of 40 h, whilst bottles read every 6 h were incubated for 42 h. Values for each substrate not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables are shown in Appendix 4.2.1.10 and 4.2.1.11 for naked oats and hay, respectively.

4.2.3.1.3 Volatile fatty acid (VFA) production

The production of VFA increased with time during incubation of naked oats and hay (Figure 4.2.5 and 4.2.6, respectively; Tables 4.2.3 and Table 4.2.4, respectively). For both naked oats and hay, there were no significant differences in total VFA production at the different reading frequencies ($p < 0.05$; Tables 4.2.3 and 4.2.4, respectively). Like DM loss, the molar percentages of VFA were recorded every 4 h for the 2 and 4 h reading frequencies and every 6 h for the 6 h reading frequency. However for clarity, only the coincident time points throughout the incubation and the final incubation time point are shown in the tables. Total VFA production throughout the incubation is shown in Figures 4.2.5 and 4.2.6, whilst Tables 4.2.3 and 4.2.4 show the statistical analysis between coincident time points for the different reading frequencies. Differences in the molar percentages of individual VFA produced during fermentation of naked oats were seen between the 4 and 6 h reading frequencies for Ac at 12 h incubation, and between the 2 and 6 h reading frequencies for Pr at 36 h incubation and Bu at 24 h incubation (Table 4.2.3).

Figure 4.2.5 Volatile fatty acid production during incubation of 1.00 g naked oats (*Avena nuda*) with a rumen microbial inoculum where gas production was recorded every 2 (-◆-), 4 (-■-) or 6 h (-▲-).

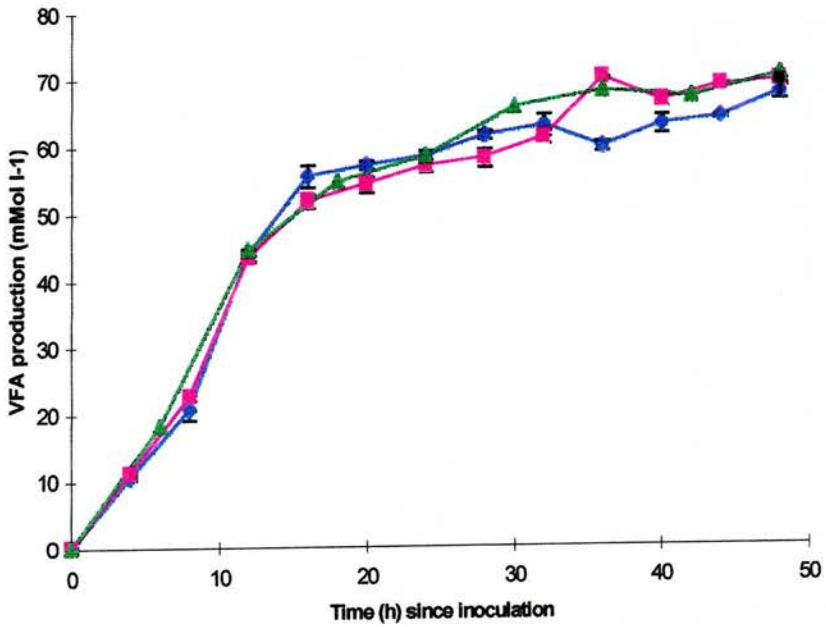
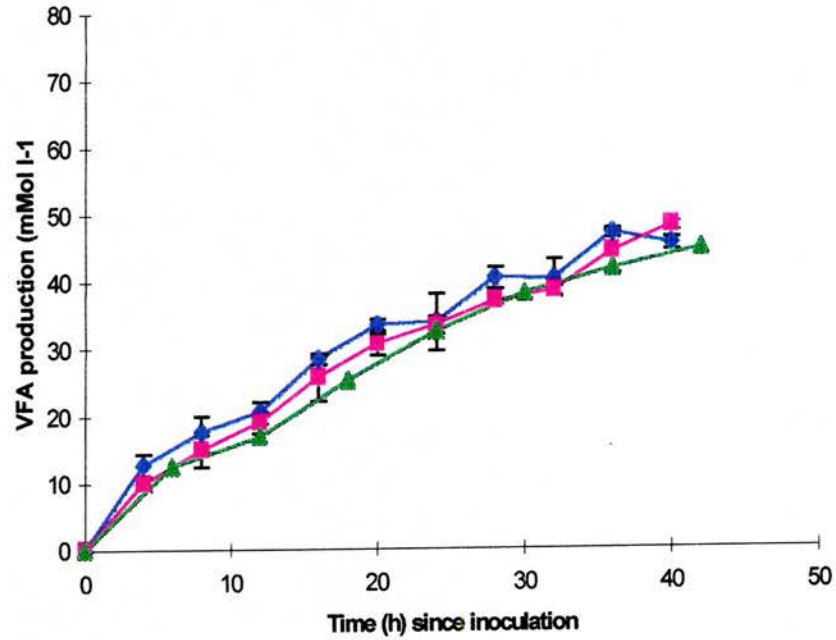


Figure 4.2.6 Volatile fatty acid production during incubation of 0.75 g perennial ryegrass (*Lolium perenne*) hay with a rumen microbial inoculum where gas production was recorded every 2 (-◆-), 4 (-■-) or 6 h (-▲-).



Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum. Each value is the mean value of three bottles. Three bottles were harvested every 4 h for volatile fatty acid (VFA) production measurements in bottles read every 2 and 4 h, whilst bottles read every 6 h were harvested for volatile fatty acid (VFA) production measurements every 6h.

However, no differences were seen between reading frequencies for the remaining VFA molar percentages at these time intervals suggesting that these differences were not biologically important. Similar differences were seen during incubation of the hay (Table 4.2.4); Ac production at 12 h being significantly higher in bottles read every 2 h compared to those read every 6 h, whilst at 36 h incubation, Ac production was significantly different between the 4 and 6 h reading frequencies. For Bu production at 12 h incubation bottles read every 6 h had a significantly higher percentage of Bu than those read every 2 and 4 h, whilst for Val differences were seen between the 2 and 4 h reading frequencies at 24 h incubation, between 2 and 6 h reading frequencies at 36 h incubation and at 40 / 42 h incubation the percentage of Val was significantly greater for the 6 h reading frequency compared to both the 2 and 4 h reading frequencies ($p < 0.05$). Again, as there were no consistent differences in the molar percentages of the other VFA these differences are unlikely to be biologically important.

Unlike the DM loss profiles, the VFA profiles for both hay and naked oats continued to increase as gas production increased. Despite differences in gas production profiles, VFA production for both naked oats and hay (Figure 4.2.5 and 4.2.6, respectively), were unaffected by the 2, 4 or 6 h reading frequencies (Tables 4.2.3 and 4.2.4, respectively).

Table 4.2.3 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced from naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum where gas production was recorded every 2, 4 or 6 h.

VFA	Incubation time (h)	Reading frequency (h) 2	4	6	s.e.d
Total (mmol l^{-1})	12	43.9^{a}	43.2^{a}	44.6^{a}	
	24	$58.4^{b,c}$	56.8^{b}	$67.3^{b,d}$	
	36	$67.7^{c,d}$	72.1^{d}	$68.0^{c,d}$	
	40/42*	$67.5^{c,d}$	70.8^{d}	70.2^{d}	5.1000
Acetate (molar %)	12	$48.37^{a,b}$	49.13^{a}	$48.16^{b,c}$	
	24	$47.68^{b,d}$	$47.41^{c,d}$	$47.81^{b,d}$	
	36	$48.07^{b,d}$	$48.03^{b,d}$	$48.40^{a,b}$	
	40/42*	$47.77^{b,d}$	$48.05^{b,d}$	47.26^{d}	0.3915
Propionate (molar %)	12	42.53^{a}	41.78^{a}	42.42^{a}	
	24	39.13^{b}	$38.36^{b,c}$	$37.81^{b,d}$	
	36	$37.85^{b,c}$	$37.16^{c,d}$	36.36^{d}	
	40/42*	$37.77^{b,d}$	$37.00^{c,d}$	$38.18^{b,c}$	0.7110
Butyrate (molar %)	12	7.34^{a}	7.39^{a}	7.78^{a}	
	24	10.54^{b}	$11.61^{b,c}$	11.92^{c}	
	36	$10.95^{b,c}$	$11.49^{b,c}$	11.98^{c}	
	40/42*	$11.06^{b,c}$	$11.57^{b,c}$	$11.09^{b,c}$	0.5630
Valerate (molar %)	12	1.75^{a}	1.71^{a}	1.63^{a}	
	24	2.51^{b}	2.61^{b}	2.45^{b}	
	36	3.13^{c}	$3.31^{c,d}$	$3.25^{c,d}$	
	40/42*	3.43^{d}	$3.38^{c,d}$	3.46^{d}	0.1315

*Bottles read every 2 or 4 h were incubated for a total of 40 h, whilst bottles read every 6 h were incubated for 42 h. Values for each parameter not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables for the above data are shown in Appendix 4.2.1.12 - 4.2.1.16.

Table 4.2.4 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced from perennial ryegrass hay (*Lolium perenne*) during incubation with a rumen microbial inoculum where gas production was recorded every 2, 4 or 6 h.

VFA	Incubation time (h)	Reading frequency (h) 2	4	6	s.e.d
Total (mmol l^{-1})	12	20.54^{a}	18.98^{a}	20.18^{a}	
	24	33.87^{b}	33.48^{b}	32.58^{b}	
	36	$47.08^{c,d}$	$44.14^{c,d}$	41.72^{c}	
	40/42*	$45.46^{c,d}$	48.06^{d}	$44.72^{c,d}$	2.9730
Acetate (molar %)	12	$61.44^{a,c}$	$60.58^{a,b}$	59.43^{b}	
	24	$63.30^{d,e}$	$62.10^{a,d}$	$62.44^{c,d,e}$	
	36	$63.28^{d,e}$	63.73^{e}	$62.00^{a,d}$	
	40/42*	$63.10^{d,e}$	$63.42^{d,e}$	$62.49^{c,d,e}$	0.7640
Propionate (molar %)	12	30.63^{a}	31.47^{a}	31.73^{a}	
	24	$27.90^{b,c}$	28.32^{b}	$27.91^{b,c}$	
	36	$26.61^{d,e}$	$25.98^{d,e}$	$27.07^{c,d}$	
	40/42*	$26.58^{d,e}$	25.65^{e}	$26.54^{d,e}$	0.5920
Butyrate (molar %)	12	6.09^{a}	6.15^{a}	6.85^{b}	
	24	$6.92^{b,c}$	$7.33^{b,c,f}$	$7.49^{c,f,g}$	
	36	$7.67^{c,d}$	$7.75^{c,d}$	$8.14^{d,e}$	
	40/42*	$7.83^{d,e,f}$	8.31^{e}	$7.95^{d,e,g}$	0.2830
Valerate (molar %)	12	$1.85^{a,b}$	1.80^{a}	$1.99^{a,b,c}$	
	24	$1.87^{a,b}$	$2.25^{c,e}$	$2.15^{b,c,d}$	
	36	$2.45^{d,e,f}$	$2.55^{e,g}$	$2.79^{g,h}$	
	40/42*	$2.49^{e,g}$	$2.62^{f,g}$	3.02^{h}	0.1558

*Bottles read every 2 or 4 h were incubated for a total of 40 h, whilst bottles read every 6 h were incubated for 42 h. Values in each section not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables for the above data are shown in Appendix 4.2.1.17 - 4.2.1.21.

4.2.3.1.4 Changes in batch culture pH

The pH profiles throughout the incubation of naked oats and hay are shown in Figures 4.2.7 and 4.2.8, respectively. For bottle series 1 (2 h reading frequency) during the incubation of 1.00 g naked oats the pH ranged from 6.4 - 6.7 units with a mean value of 6.5 ± 0.12; the range of pH for series 2 (4 h reading frequency) was 6.3 - 6.7 units with a mean value of 6.5 ± 0.13, whilst the pH for series 3 (6 h reading frequency) ranged from 6.3 - 6.7 with a mean value of 6.5 ± 0.14. During the incubation of hay the pH in series 1 ranged from 6.6 - 6.8 with a mean value of 6.7 ± 0.04, the range of pH for series 2 was 6.6 - 6.8 with a mean value of 6.7 ± 0.05 and for series 3 the range of pH was 6.6 - 6.8 units with a mean value of 6.7 ± 0.06. The pH profile throughout the incubation was similar for all series of bottles, during the incubation of both naked oats and hay (Table 4.2.5; Figure 4.2.7 and 4.2.8, respectively).

Table 4.2.5 pH during incubation of 1.00 g DM naked oats (*Avena nuda*) or 0.75 g DM perennial ryegrass (*Lolium perenne*) hay at 12, 24, 36 and 40 / 42 h incubation with a rumen microbial inoculum, where gas production was recorded every 2, 4 or 6 hours.

Substrate	Reading frequency (h)	Incubation time (h) 12	24	36	40 / 42*	s.e.d
Naked oats	2	$6.45^{a,b}$	$6.46^{b,c}$	6.54^{e}	6.50^{d}	
	4	$6.44^{a,b}$	$6.45^{a,b}$	$6.51^{d,e}$	$6.49^{c,d}$	
	6	6.42^{a}	$6.44^{a,b}$	$6.51^{d,e}$	$6.49^{c,d}$	0.03868
Hay	2	6.66^{a}	$6.72^{c,d,e}$	$6.76^{e,f}$	$6.73^{c,d,e}$	
	4	$6.67^{a,b}$	$6.71^{b,d}$	$6.76^{e,f}$	6.78^{f}	
	6	6.66^{a}	$6.75^{d,f}$	6.79^{f}	$6.70^{a,b,c}$	0.04430

*Bottles read every 2 or 4 h were incubated for a total of 40 h, whilst bottles read every 6 h were incubated for 42 h. Values for each substrate not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables are shown in Appendix 4.2.1.22 and 4.2.1.23 for naked oats and hay, respectively.

Figure 4.2.7 pH during incubation of 1.00 g naked oats (*Avena nuda*) with a rumen microbial inoculum where gas production was recorded every 2 (-◆-), 4 (-■-) or 6 h (-▲-).

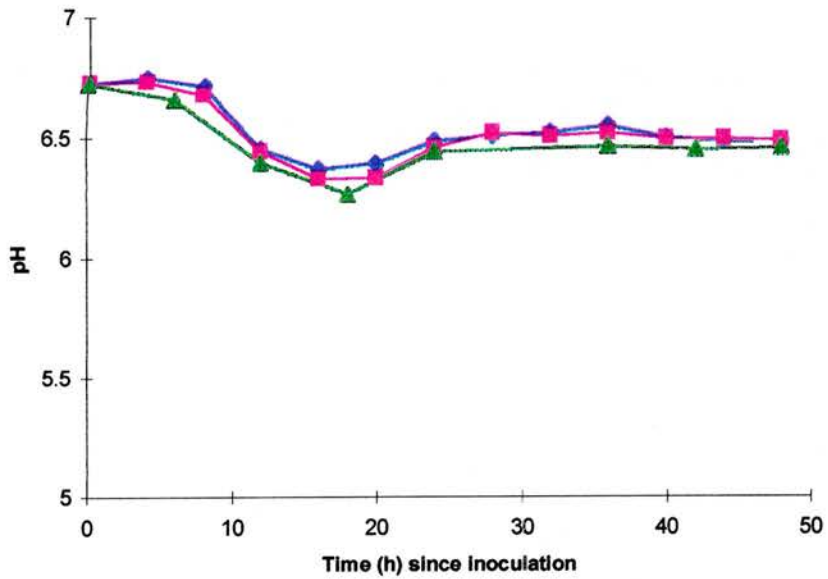
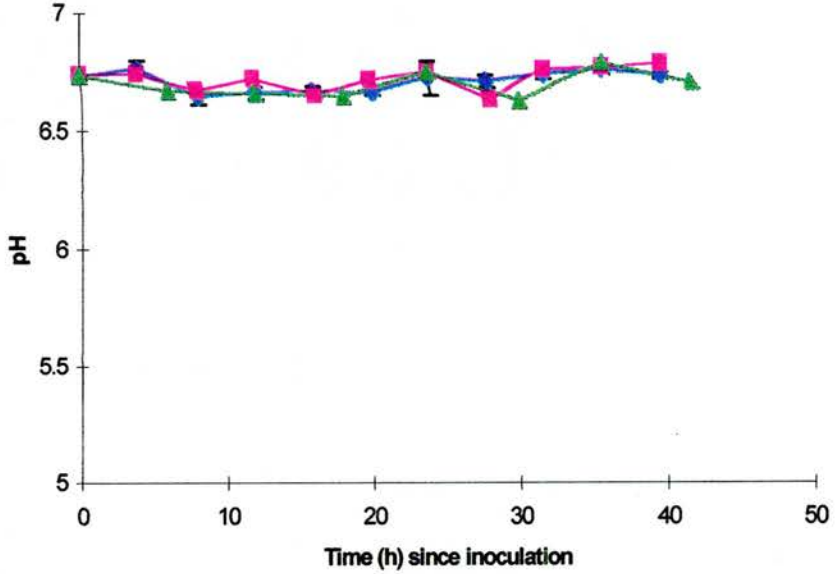


Figure 4.2.8 pH during incubation of 0.75 g perennial ryegrass (*Lolium perenne*) hay with a rumen microbial inoculum where gas production was recorded every 2 (-◆-), 4 (-■-) or 6 h (-▲-).



Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum. Each value is the mean value of three bottles. Three bottles were harvested every 4 h for pH measurements in bottles read every 2 and 4 h, whilst bottles read every 6 h were harvested for pH measurements every 6h.

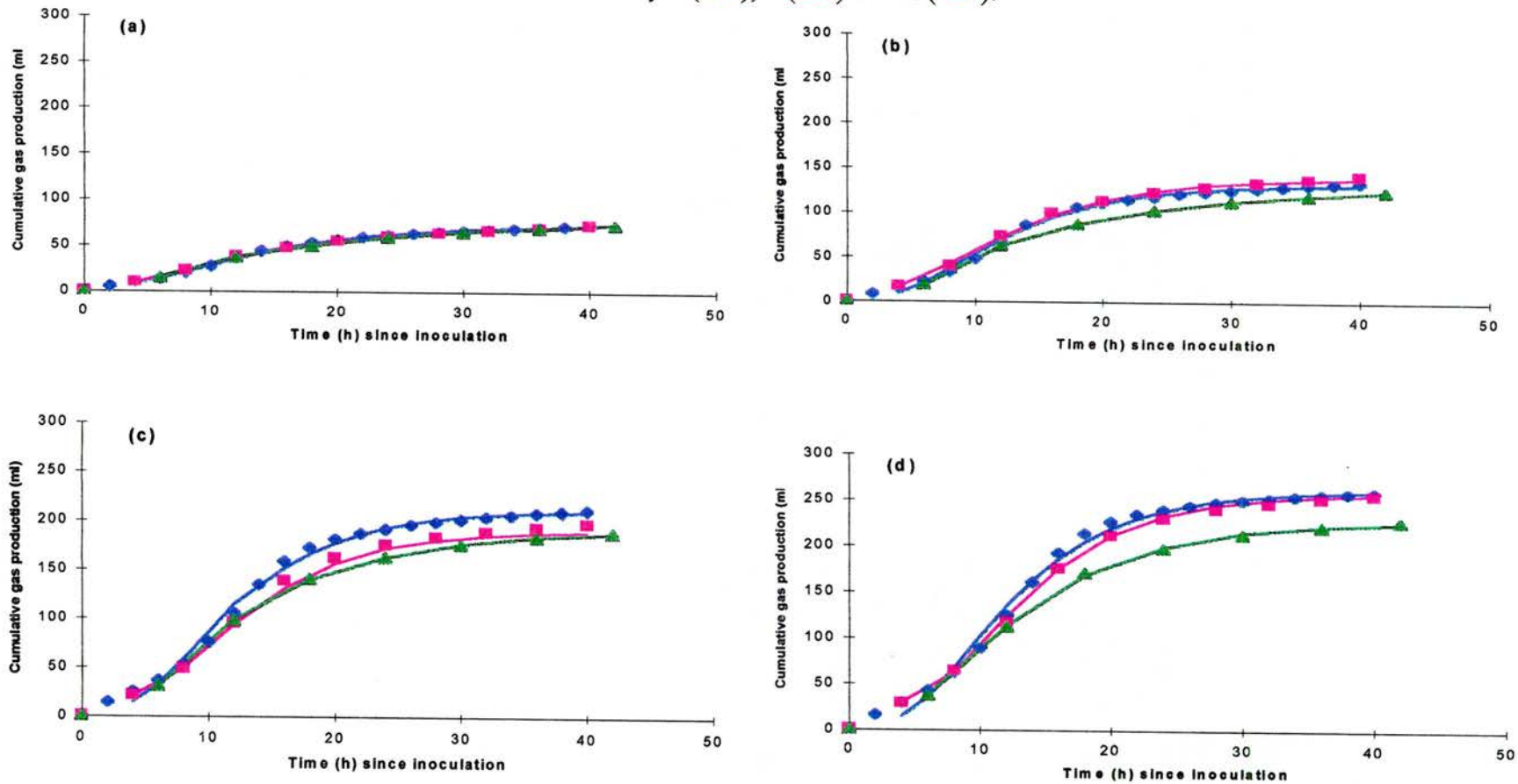
4.2.3.2 *The effect of different quantities of substrate (0.25, 0.50, 0.75 and 1.00 g naked oats) (Experiment 4.2.3)*

4.2.3.2.1 Gas production

The gas production profiles from bottles containing 0.25, 0.50, 0.75 and 1.00 g naked oats and read every 2, 4 or 6 h are shown in Figure 4.2.9. For each reading frequency gas production was greatest with 1.00 g naked oats with proportionately less gas being produced from the smaller substrate weights. Parallel curve analysis of the data in the profiles (Appendix 4.2.2) was carried out in two ways. Firstly, the substrate weights were compared at each reading frequency. This indicated that there were no significant differences in the specific rate of gas production between bottles containing different substrate weights (0.25, 0.50, 0.75 and 1.00 g naked oats) when the bottles were read either every 2 or every 4 h. However, when bottles were read every 6 h significant differences in the rate of gas production were seen between the different substrate weights ($p < 0.01$). The total cumulative volume of gas produced was significantly different ($p < 0.001$) between the different substrate weights at all reading frequencies. The largest quantity of gas was produced by 1.00 g naked oats, followed by 0.75 g, 0.50 g and 0.25 g naked oats.

Secondly, parallel curve analysis of the gas production profiles from fermentation of each of the different substrate weights was compared across the different reading frequencies (for example, the gas production profile obtained during incubation of 0.25 g with a 2 h reading frequency was compared with the gas production profiles obtained for 0.25 g using a reading frequency of 4 or 6 h; Appendix 4.2.2.2). The total cumulative gas volume from fermentation of 0.25 g was found to be similar for all reading frequencies (2, 4 and 6 h), however the rate of gas production was significantly different ($p < 0.05$). The gas production profiles from 0.50 g naked oats showed significant differences in both the total cumulative gas volume ($p < 0.001$) and the rate of gas production ($p < 0.05$) between the different reading frequencies (2, 4 and 6 h). For both 0.75 and 1.00 g naked oats no significant differences were found between the rates of gas production at reading frequencies of 2, 4 or 6 h. However,

Figure 4.2.9 Cumulative gas production during incubation of **(a)** 0.25, **(b)** 0.50, **(c)** 0.75 and **(d)** 1.00 g naked oats (*Avena nuda*) with a rumen microbial inoculum where gas production was recorded every 2 (-◆-), 4 (-■-) or 6 h (-▲-).



Each value represents the mean of three bottles, whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure. Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum.

total cumulative gas volume differed significantly with the different reading frequencies ($p < 0.001$).

Fitted parameters and derived quantities for the gas production profiles are shown in Table 4.2.6. The rates of gas production, b and c, ranged from 0.0169 h^{-1} (during incubation of 0.25 g naked oats using a reading frequency of 6 h) to 0.2776 h^{-1} (during incubation of 1.00 g naked oats using a reading frequency of 4 h) and from -1.1526 $h^{-0.5}$ (during incubation of 1.00 g naked oats using a reading frequency of 4 h) to 0.2649 $h^{-0.5}$ (during incubation of 0.25 g naked oats using a reading frequency of 6 h), for b and c respectively. The mean value for b across all reading frequencies and substrate weights was 0.1779 ± 0.02628 h^{-1}, whilst that for c was - 0.5543 ± 0.14257 $h^{-0.5}$. The largest total cumulative volume of gas, A, was produced during incubation of 1.00 g naked oats where gas production was recorded every 2 h (261 ml) whilst least gas was produced during incubation of 0.25 g naked oats, at a reading frequency of 2 h (73 ml). The mean value for A across all reading frequencies and substrate weights was 166 ± 19.2 ml. The lag time, L_T, ranged from 0.88 h (during incubation of 0.25 g using a 4 h reading frequency) to 4.34 h (during incubation of 0.50 g using a 6 h reading frequency), with a mean value of 3.26 ± 0.271 h. Values for t_{50} and t_{95} ranged from 11.49 h (during incubation of 0.75 g using a 2h reading frequency) to 14.33 h (during incubation of 0.25 g using a 6 h reading frequency) and from 27.21 h (for 0.75 g using a 2 h reading frequency) to 75.68 h (during incubation of 0.25 g with a 6 h reading frequency) with mean values of 12.19 ± 0.234 h and 36.32 ± 4.193 h for t_{50} and t_{95}, respectively.

The large values of t_{95} for bottles containing both 0.25 and 0.50 g naked oats which were read every 6 h was unexpected as from Figure 4.2.9 there does not appear to be any differences between the profiles for the 2, 4 or 6 h reading frequency for these substrate weights. To see if this was as a result of the fewer data points available for fitting the France *et al.* (1993) model to the gas production profiles (i.e. a reading frequency of 2 h gives 20 cumulative gas production data points whilst a 6 h reading frequency only gives 7 data points for the incubation) the 2 h data were recalculated

using only the 6 h frequency time points. Refitting the data to the France *et al.* (1993) model resulted in values of t_{95} of 41.59 ± 0.951 h and 28.42 ± 1.625 h, for 0.25 and 0.50 g of naked oats respectively. Therefore, in fermentations involving low substrate weights and long reading frequencies values for t_{50} and t_{95} may be increased, but not as a consequence of alterations in the fermentation, merely due to the shape of the curve imposed by the number of data points.

Table 4.2.6 The gas production parameters (France *et al.*, 1993); rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for 0.25, 0.50, 0.75 and 1.00 g naked oats incubated with a rumen microbial inoculum in the pressure transducer technique, where gas production was recorded every 2, 4 or 6 h.

Gas production parameters	Reading frequency (h)	Substrate weight (g) 0.25	0.50	0.75	1.00	sed
b (h^{-1})	2	0.1752^{a}	0.2379^{b}	$0.2534^{b,c}$	$0.2587^{c,d}$	
	4	0.1058^{e}	0.2071^{f}	$0.2678^{c,d}$	0.2776^{d}	
	6	0.0169^{g}	0.0343^{g}	0.1105^{e}	$0.1900^{a,f}$	0.0095
c ($h^{-0.5}$)	2	-0.5799^{a}	-0.8383^{b}	$-0.9167^{b,c}$	-0.9566^{c}	
	4	-0.1988^{d}	-0.7175^{e}	-1.0767^{f}	-1.1526^{f}	
	6	0.2649^{g}	0.2557^{g}	-0.1146^{d}	$-0.6202^{a,e}$	0.0480
A (ml)	2	72.7^{a}	132.1^{b}	210.6^{c}	261.2^{d}	
	4	74.1^{a}	139.4^{b}	189.5^{e}	257.5^{d}	
	6	100.1^{f}	136.4^{b}	192.3^{e}	227.6^{g}	7.18
B	2	40.4^{a}	$59.6^{a,b}$	$86.1^{b,c}$	$102.3^{c,d}$	
	4	$67.4^{a,b}$	$68.2^{a,b,c}$	$57.1^{a,b}$	$69.2^{a,b,c}$	
	6	173.1^{e}	272.2^{f}	235.7^{g}	130.5^{d}	16.85
L_T (h)	2	2.74^{a}	$3.11^{a,b}$	$3.29^{b,c}$	$3.42^{b,c,d}$	
	4	0.88^{e}	$2.98^{a,c}$	4.04^{f}	4.31^{f}	
	6	$3.44^{b,c,d}$	4.34^{f}	$3.87^{d,f}$	2.66^{a}	0.2320
t50 (h)	2	12.088^{a}	11.549^{a}	11.490^{a}	11.725^{a}	
	4	12.181^{a}	11.670^{a}	12.091^{a}	$12.365^{a,b}$	
	6	14.333^{c}	13.139^{b}	11.646^{a}	12.026^{a}	0.4307
t95 (h)	2	$32.68^{a,b}$	27.87^{a}	27.21^{a}	27.29^{a}	
	4	39.26^{c}	$29.93^{a,b}$	27.98^{a}	28.05^{a}	
	6	75.68^{d}	53.78^{e}	$35.11^{b,c}$	$31.01^{a,b}$	3.125

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 4.2.9. Values for each parameter not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables are shown in Appendix 4.2.2.3 - 4.2.2.9.

4.2.3.3 *The effect of altering the duration of reading (20, 60 or 120 s) (Experiment 4.2.4)*

4.2.3.3.1 Gas production

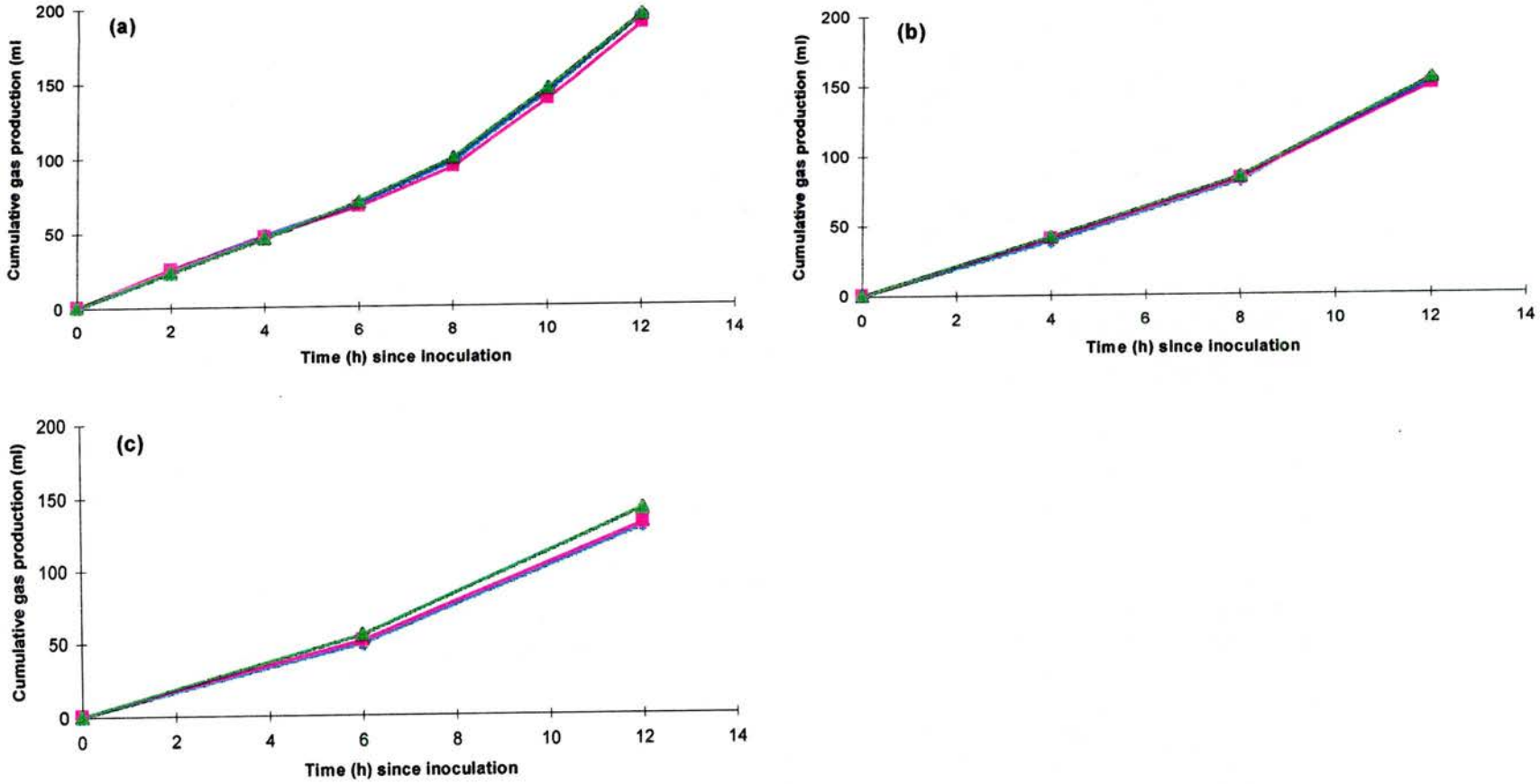
The resulting gas production profiles for bottles read at 2, 4 and 6 h frequencies are shown in Figure 4.2.10. Three sets of bottles were inoculated for each reading frequency and the duration of the gas production readings in each case were 20, 60 or 120 s. This experiment involved short-term incubations of not more than 12 h duration, and the data was not therefore fitted to the model of France *et al.* (1993). The total amount of gas produced in 12 h in each treatment is shown in Table 4.2.7. Analysis of variance (Appendix 4.2.4) showed that cumulative gas production was significantly greater in bottles read every 2 h, than in bottles read every 4 h, which in turn, produced significantly more gas than those read every 6 h ($p < 0.001$). This result was as expected from the previous experiments. The effect of increasing the duration of reading (reading time = the time the pressure transducer is in contact with the head-space of the culture bottles) from 20 s to 120 s had no significant effect on gas production where bottles were read every 2 or 4 h. However, for bottles which were read every 6 h, increasing the reading time to 120 s resulted in the production of significantly more gas (141 ml as opposed to 129 or 130 ml; Table 4.2.7) than when reading times of 20 s or 60 s were used ($p < 0.05$) (Figure 4.3.10 c).

Table 4.2.7 The cumulative volume of gas produced during incubation of 1.00 g naked oats with rumen a microbial inoculum, using different reading frequencies (2, 4 or 6 h) and allowing the pressure transducer assembly to remain in the bottle for 20, 60 or 120 s during each reading.

Duration of reading	Reading frequency		
(s)	2 h	4 h	6 h
20	193.7^{a}	152.6^{b}	128.9^{c}
60	188.1^{a}	149.2^{b}	130.4^{c}
120	194.3^{a}	154.1^{b}	141.2^{d}

Values not bearing the same superscript differ significantly ($p < 0.01$); sed = 2.65. The analysis of variance table is shown in Appendix 4.2.4.

Figure 4.2.10 Cumulative gas production during incubation of 1.00 g naked oats (*Avena nuda*) with a rumen microbial inoculum, where gas production was recorded every **(a)** 2, **(b)** 4 or **(c)** 6 h and the pressure transducer assembly was allowed to remain in the bottle for 20 (-◆-), 60 (-■-) or 120 s (-▲-).



Each value represents the mean of three bottles, in all cases SE were less than 5 % of the values shown in the figure. Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum.

4.2.4 Discussion

Gas production profiles obtained using the pressure transducer technique, for both a rapidly degradable substrate (naked oats) and a slowly degradable substrate (ryegrass hay), were significantly affected by the reading frequency (the interval between consecutive readings), i.e. the time allowed for pressure to increase in the head-space. However, DM loss and VFA production (VFA molar proportions) were not affected by the different reading intervals, suggesting that under the conditions employed these fermentations were not end-product inhibited by accumulating gases and that the fermentation stoichiometry did not change in response to an increase in head-space gas pressure. The most likely explanation for the results obtained is that in bottles with increased pressure in the head-space, the equilibrium between the gas dissolved in and that liberated by the medium was altered, resulting in an increase in the volume of gas which was absorbed into the culture medium, in accordance with Henry's Law (section 2.2.7.3.1). In addition the dwell time used to read the bottles may have been too short at the higher reading intervals to measure all the gas coming from solution.

Parallel curve analysis (section 4.2.3.2.1) indicated that there was an interaction between the reading frequency and the amount of substrate incubated. Reading frequency had a significant effect on the volume of gas produced in bottles containing 0.50, 0.75 and 1.00 g naked oats ($p < 0.001$). However, as 0.25 g results in the production of less gas no significant differences in gas volume were seen between the 2, 4 or 6 h reading frequencies when 0.25 g naked oats was incubated. Based on these results, using 0.25 g substrate in the pressure transducer technique would allow a reading frequency of 6 h to be utilised hence reducing overnight surveillance and the intensity of labour involved in these studies. However, although using 0.25 g substrate would reduce the intensity of labour involved, the small volumes of gas produced may make small differences between feedstuffs more difficult to detect, hence 0.50 or 0.75 g substrate may be preferable.

Where significant differences in gas production were seen between reading frequencies, the volume of gas produced from bottles read every 4 or 6 h tended towards that produced from bottles read every 2 h towards the end of the incubation. This suggests that the gas absorbed by the medium under high pressure, diffused back into the head-space when the pressure was relieved. It also suggests that when significant quantities of gas were dissolved in the liquid phase, i.e. during rapid fermentation, the pressure transducer was in contact with the head-space for insufficient time to allow all the gas time to diffuse back into the head-space and be measured. However, allowing the pressure transducer assembly to dwell in the bottle head-space for 20, 60 or 120 s had no effect on the volume of gas recorded when the bottles were read every 2 or 4 h. Whilst, allowing the pressure transducer assembly to dwell in the head-space for 120 s when bottles were read every 6 h resulted in the measurement of an increased volume of gas compared to dwell times of 20 or 60 s. However, the increased volume of gas recorded was small (approximately 11 ml in 12 h) and still significantly lower than that observed with reading intervals of 2 and 4 h. This suggests that either the gas requires a longer period of time to diffuse from the culture medium into the head-space or that some of the gas became 'trapped' in the medium. One reaction which may remove CO_2 from the medium is via the formation of NH_4HCO_3 from HCO_3^- ions (from the solubilisation of CO_2) and NH_4^+ ions (either present in the culture medium or produced from the deamination of plant and microbial protein);

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^- \text{ which may then combine with } NH_4^+;$$
$$NH_4^+ + HCO_3^- \leftrightarrow NH_4HCO_3$$

If CO_2 was removed from the system in this way, the volume of gas produced during the 4 and 6 h reading intervals will be less than that produced when a 2 h reading interval is employed. However, this explanation may be unlikely as the medium already contains a large number of ions. Therefore a change in the rate of release of CO_2 from the medium is the most likely explanation for the results obtained (Dr. A. Harrison, Edinburgh University - personal communication). The increase in reading

time only partially corrected the problem encountered with increased head-space pressure. Diffusion of gas out of solution into the head-space is likely to be related to the surface area of the gas / liquid interface, therefore, different bottle shapes could be used to increase the rate at which the gas will diffuse out of solution.

Information regarding the effect of increasing head-space pressure on gas production readings is scarce. However, Schofield and Pell (1995a) have conducted a similar study whereby the volume available for gases to collect was increased; effectively reducing head-space pressure. Their study involved conducting fermentations in serum bottles with a nominal volume of 50 ml. The total volume of the system was then increased to give volumes of 100 or 150 ml, by attaching either 50 or 100 ml serum bottles to the incubation vessel with rubber tubing. This resulted in three volume treatments; 50 ml (no bottle attached), 100 ml (50 ml bottle attached) and 150 ml (100 ml bottle attached). At the end of the incubation (48 h) they found no significant differences in total gas production, NDF digestibility or VFA production between volume treatments. The discrepancy between gas volumes in the current study and those obtained by Schofield and Pell (1995a) may be explained by the method used to record gas production. In the technique used by Schofield and Pell (1995a) gas production was measured by sensors as a voltage reading. This voltage was then converted to a volume measurement using a correction factor which accounted for solubilised CO_2 (by incorporating Henry's Law) and for the total volume of the system. Their calculation therefore accounted for gas which was solubilised in the medium whilst the actual amount of gas in the head-space of culture bottles was measured in the pressure transducer technique. The conversion factor adopted by Schofield and Pell (1995a) could not be adopted for the present study as the amount of CO_2 in the aqueous phase was continually fluctuating as gas was allowed to accumulate and then released at regular intervals throughout the incubation. Whereas in the study conducted by Schofield and Pell (1995a) the bottles were not vented and the amount of CO_2 present in the liquid phase was reported to increase linearly throughout the incubation.

It is therefore important to consider the reading frequency during gas production studies using the pressure transducer technique, in order that the pressure exerted on the fermentation is the same for all substrates. One method of ensuring that all bottles are subjected to similar conditions throughout the fermentation is to vent the bottles at a predetermined pressure, as is employed in the APES (Davies *et al.*, 1995) and other automated systems.

4.3 The effect of shaking versus not shaking on the production of gas from batch cultures inoculated with rumen micro-organisms

4.3.1 Introduction

During digestion in the rumen contraction and relaxation of the muscular wall acts to mix the digestive juices and micro-organisms with food particles (Wyburn, 1980; McDonald *et al.*, 1995). There are conflicting reports on whether or not the bottle contents should be shaken during gas production studies. For example, Menke and Steingass (1988), Pell and Schofield (1993) and Cone *et al.* (1996) employ shaking devices in their gas production techniques (section 2.1.4.3), whilst Davies *et al.* (1995) do not. In the technique of Theodorou *et al.* (1994) the bottles are shaken after every gas reading, but not continually throughout the incubation. According to Pell and Schofield (1993), shaking the bottles is an important step in the fermentation which avoids supersaturation of the medium with CO_2. However, supersaturation of the medium may only be a problem where gas is allowed to accumulate and is not removed during the fermentation. The aim of this experiment was to investigate the effect of shaking on the resulting gas production profiles, and hence determine whether shaking is an important step in gas production as determined by the pressure transducer technique.

4.3.2 Materials and Methods

Three identical series of bottles (series 1, 2 and 3) were used. All bottles contained 0.75 g perennial ryegrass hay (*Lolium perenne*) (Appendix 1) and were inoculated with 10 ml of microbial inoculum (prepared from digesta collected from a rumenally - fistulated, hay - fed sheep) and incubated in a constant temperature room (39 °C). Bottles were either shaken continuously (series 1), shaken after every gas reading (as in a 'normal' gas production study using the PTT; series 2) or not shaken (series 3). All treatments began immediately after inoculation, at time 0 h. All bottles were read at 2, 4, 6, 8, 10, 12, 14, 16, 18, 24, 30, 36, 42, 48, 54, 60 and 72 h after inoculation. The shaken bottles (series 1) were placed on a Griffin Orbital Shaker (Fisher Scientific UK Ltd.), and shaken at 115 rpm. They were shaken continuously

throughout the incubation the shaker only being turned off to read the bottles (approximately 4 min at every reading interval). Bottles in series 2 were shaken after every gas reading as per usual in the PTT, that is, they were shaken by hand for approximately 3 s each. Bottles in series 3 (not shaken) remained stationary throughout the 72 h incubation period. Three bottles were included as blanks for each series.

At the end of the incubation, pH was recorded, samples were taken for VFA analysis and DM loss was determined as described in sections 3.10 and 3.12.1.

The experiment was a factorial design consisting of 3 shaking treatments and 3 replicate bottles (3 x 3). The gas production profiles were fitted to the model of France *et al.* (1993) and compared using the parallel curve analysis function of MLP (Ross, 1987) (section 3.11). Values for the modelled gas production parameters, DM loss, VFA production and pH were analysed using analysis of variance in Genstat 5 (Lawes Agricultural Trust, 1993). Differences between treatments were calculated using LSD (section 3.11).

4.3.3 Results

4.3.3.1 Gas production

The gas production profiles for the different treatments are shown in Figure 4.3.1. Parallel curve analysis indicated that total gas production ($p < 0.001$) and the rate of gas production ($p < 0.01$) were significantly higher in the stationary bottles (series 3) compared to the continuously and intermittently shaken bottles (series 1 and 2, respectively) (Appendix 4.3.1). The gas production parameters (France *et al.*, 1993) for each treatment are shown in Table 4.3.1. Small but significant differences were detected by analysis of variance between parameter values for the different treatments and these are detailed in Table 4.3.1. The rates of gas production, b and c, ranged from 0.0442 h^{-1} for the stationary bottles to 0.0593 h^{-1} for the continuously shaken bottles and from -0.1288 $h^{-0.5}$ for the continuously shaken bottles to -0.0270 $h^{-0.5}$ for the stationary bottles, respectively. The mean value for b for all shaking treatments

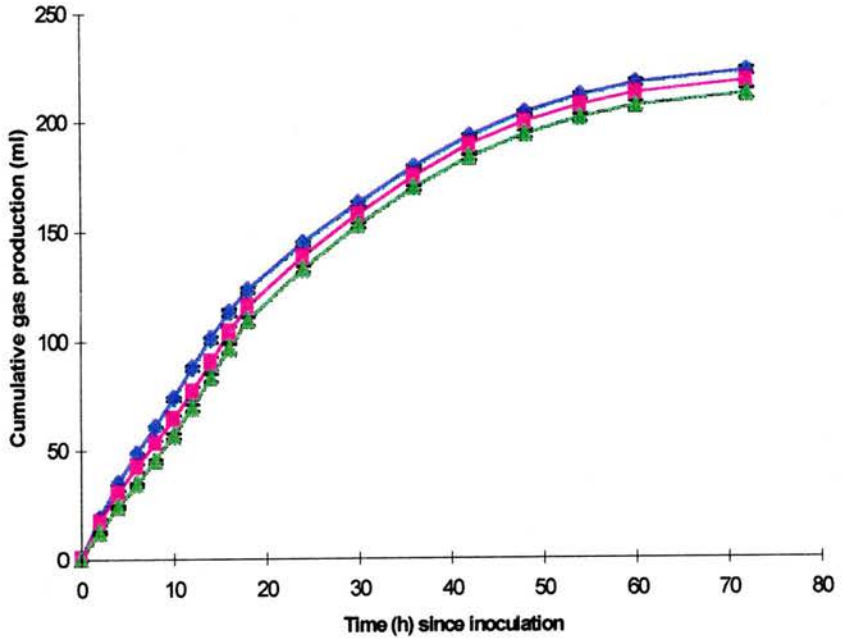
was 0.0524 ± 0.00044 h^{-1} whilst that for c was -0.0839 ± 0.02999 $h^{-0.5}$. Total gas production was greatest in the stationary bottles (236 ml) and least in the continuously shaken bottles (221 ml). The mean total gas production for all treatments was 228.8 ± 4.30 ml. The lag time, L_T (h), encountered prior to active fermentation ranged from 0.09 h for the stationary bottles to 1.18 h for the continuously shaken bottles, with a mean value across all treatments of 0.69 ± 0.318 h. The time taken to produce 50 or 95 % of the total gas production (t_{50} and t_{95}, respectively) was shortest when bottles were not shaken (17.6 and 65.8 h for t_{50} and t_{95}, respectively) whilst the bottles which were continuously shaken produced the longest value for t_{50} (19.0 h) and the intermittently shaken bottles the longest value for t_{95} (68.6 h). The mean values for t_{50} and t_{95} were 18.4 ± 0.44 h and 66.8 ± 0.88 h, respectively.

Table 4.3.1 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for 0.75 g perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum in the pressure transducer technique and shaken continuously (series 1), after every gas production reading (series 2) or not shaken (series 3) throughout the 72 h incubation.

Gas production parameters	Shaking treatment			sed	Significance
	series 1	series 2	series 3		
b (h^{-1})	0.0593^a	0.0536^b	0.0442^c	0.0017	*
c ($h^{-0.5}$)	-0.1288^a	-0.0960^b	-0.0270^c	0.0025	***
A (ml)	221.3^a	$228.9^{a,b}$	236.2^b	2.94	**
B	194.9^a	205.0^b	228.6^c	2.54	*
L_T (h)	1.18^a	0.80^b	0.09^c	0.0453	**
t50 (h)	19.05^a	$18.58^{a,b}$	17.56^b	0.517	*
t95 (h)	66.15	68.61	65.81	1.880	NS

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 4.3.1. Values in rows not bearing the same superscripts differ significantly (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The analysis of variance tables are shown in Appendix 4.3.2 - 4.3.8.

Figure 4.3.1 Cumulative gas production during incubation of perennial ryegrass (*Lolium perenne*) hay with a rumen microbial inoculum, where bottles were subjected to continual shaking (-▲-), shaking after every gas production reading (-■-) or not shaken (-◆-) throughout the incubation.



Each value represents the mean of three bottles, whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure. Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum.

4.3.3.2 Dry Matter Loss

More substrate was lost to degradation in the continuously shaken bottles (800.8 mg g^{-1}) compared to in the intermittently shaken bottles (771.3 mg g^{-1}; $p < 0.05$), which in turn had significantly more substrate loss than bottles which were not shaken (745.7 mg g^{-1}; $p < 0.05$). The analysis of variance table is shown in Appendix 4.3.9.

4.3.3.3 Volatile fatty acid (VFA) production

The production of VFA from series 1, 2 and 3 are shown in Table 4.3.2. There were no significant differences in VFA production between the different shaking regimes (Appendix 4.3.10 - 4.3.14). Each series producing approximately 30 mmol l^{-1} of Ac, 15 mmol l^{-1} of Pr, 5 mmol l^{-1} Bu and 1.5 mmol l^{-1} Val. The VFA molar percentages were also not affected giving a mean value of 57 Ac: 30 Pr: 10 Bu: 3 Val for all series of bottles.

Table 4.3.2 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate and valerate, produced from perennial ryegrass hay (*Lolium perenne*) during incubation with a rumen microbial inoculum in the pressure transducer technique and shaken continuously (series 1), after every gas production reading (series 2) or not shaken (series 3) throughout the 72 h incubation.

	Treatment				
	Series 1	Series 2	Series 3	sed	sig.
Total VFA (mmol l^{-1})	53.30	50.00	51.20	3.290	NS
Acetate (molar %)	30.31	28.59	29.20	2.002	NS
Prpionate (molar %)	15.32	14.81	15.43	0.869	NS
Butyrate (molar %)	5.97	5.21	5.11	0.329	NS
Valerate (molar %)	1.68	1.36	1.48	0.153	NS

The analysis of variance tables for the above data are shown in Appendix 4.3.10 - 4.3.14.

4.3.3.4 Changes in batch culture pH

The pH after the 72 h incubation of hay was significantly higher in the bottles in series 1 (6.5 ± 0.02 pH units), compared to those in both series 2 and 3; 6.6 ± 0.03

and 6.6 ± 0.01, respectively ($p < 0.05$; Appendix 4.3.15). However, this difference of 0.1 pH unit is not likely to be of biological significance.

4.3.4 Discussion

Movement in the rumen occurs through contraction and relaxation of the rumen wall (Wyburn, 1980; McDonald *et al.*, 1995). Quin and van der Wath (1938) reported the frequency of contractions in Merino sheep, recorded during the morning and before feeding, to be between 5 to 7 ruminal contractions per 5 min period, this increased to 20 - 25 movements per 5 min during feeding. The force and magnitude of these contractions was found to vary considerably ranging from a pressure of - 10 mm.H_2O during quiescence to an average of + 70 mm.H_2O pressure at the height of ruminal contractions. The rhythm of the movements also varied from an evenly spaced rhythm in which all the contractions were of approximately the same magnitude to one in which both the rate and size of the movements became irregular. Thus it is unlikely that continual shaking over a 72 h period at a fixed rate will mimic ruminal contractions exactly.

Gas production tended to be higher in the bottles which were not shaken, or shaken intermittently (after every reading) compared to those that were shaken continuously. A possible explanation for this may be that the micro-organisms could not establish on the feed particles in shaken bottles as effectively as those which were in the intermittently shaken bottles or bottles which were not shaken at all. The presence of gas bubbles which will develop on feed particles when the bottles are shaken may also be responsible, in part, for the decrease in gas production. Gas bubbles on the food particles will reduce the surface area of the food particle available for microbial attachment making it more difficult for micro-organisms to establish and hence degrade the food particle (Bowman & Firkins, 1993; Chesson, 1993). Johnson (1966) found that violent agitation of the culture medium during *in vitro* studies reduced the digestion of cellulose. However, in the current study DM loss appeared to be greater in the continuously shaken bottles. One explanation for this discrepancy may be that the action of the shaking movement caused a physical reduction in particle size hence

increasing the quantity of particles which were small enough to pass through the crucible and which were therefore considered to be fermented. If the micro-organisms could not establish on the feed particles in the continuously shaken bottles as effectively as those which were in the intermittently shaken bottles or bottles which were not shaken, reduced microbial contamination of the feed residues may also help to explain this difference. There may have been less microbial contamination of the feed residues compared to the other treatments, thus accounting for the higher apparent DM loss of the continuously shaken hay (due to lower microbial contamination). However, the VFA production was not different between treatments implying that microbial activity was similar, hence, gas production should not have differed between treatments. The measurement of less gas may, therefore, be simply explained through more CO_2 dissolving in the medium via the increased gas - liquid interface produced by the shaking movement. The effect of gas pressure on gas solubility described by Henry's Law is commented on in the previous section. It is not inconceivable therefore that an increased liquid surface area, as caused by the shaking movement, will result in more gas passing into solution. Further work is therefore needed to investigate the effect of increased surface area on gas production profiles during *in vitro* gas production studies.

Rymer *et al.* (1997b) noted the opposite effect of shaking on gas production; recording a linear increase in gas production with an increasing shaking speed of 0 - 45 strokes per minute. However, their study was fundamentally different to the one reported here in that they did not have any substrate nor microbial inocula in the culture bottles. Therefore, their system represented only part of the process and they were essentially describing a physical phenomenon, not associated with any biological process.

CHAPTER 5 - BIOLOGICAL FACTORS AND THE EFFECT OF FEED CHEMISTRY AND THE SOURCE OF THE MICROBIAL INOCULUM ON GAS PRODUCTION STUDIES

In addition to physical factors, biological and chemical factors may also influence gas production. For example, the chemical nature of the feedstuff, the composition of the culture medium, the inoculum source (for example, rumen digesta or faeces) and the diet of the donor animal will all affect the resulting gas production profiles. The aim of this chapter was therefore to investigate some of the biological and chemical factors which affect gas production. The factors investigated were feed chemistry and its influence on gas and VFA production (section 5.1), gas production from the soluble and insoluble fractions of naked oats and hay (section 5.2), and the effect of the source of the inoculum (section 5.3).

5.1 Feed chemistry and its effect on the resulting volatile fatty acid (VFA) and gas production profiles from batch cultures inoculated with rumen micro-organisms

5.1.1 Introduction

Starch in cereals, fructans in grasses and the carbohydrate portion of dietary fibre (the non-starch polysaccharides - NSP) are often the major sources of dietary energy in feedstuffs for ruminants. Their fermentation by rumen micro-organisms results in the production of volatile fatty acids (VFA) and gas (predominately CO_2 and CH_4) (Hungate, 1966). The major VFA are acetic (Ac), propionic (Pr) and butyric (Bu) acids. The extent and relative proportions in which the major VFA are produced by the rumen microbial population depends upon the fermentation stoichiometry which is largely related to the nature of the feedstuff. Feedstuffs with a low starch and high fibre content tend to produce VFA in the molar ratio 75 Ac: 20 Pr: 5 Bu, whereas those high in starch but low in fibre tend towards VFA molar ratios of 50 Ac: 35 Pr: 15 Bu (McDonald *et al.*, 1995).

Measurement of the gas produced during the incubation of a feedstuff with buffered rumen fluid, *in vitro*, allows the *in vivo* digestibility (fermentability) of that feedstuff to be estimated (Menke *et al.*, 1979; section 2.1.4.3). The gas produced is a result of both the formation of gaseous fermentation end-products (i.e. CO_2, H_2 and CH_4; fermentation gases), and CO_2 released from the predominately bicarbonate buffered culture medium during the production of VFA (i.e. acidification gas). The amount of gas produced due to the fermentation is dependent upon the VFA molar proportions produced (Hungate, 1966; section 2.2.8.1). For example, the formation of butyric acid arises from the condensation of two molecules of acetic acid to form acetoacetic acid, which is then reduced to butyric acid with four atoms of hydrogen. Every mole of butyric acid produced results in the formation of two moles of CO_2 plus two moles of H_2 (equation 2.23, chapter 2). Acetic acid arises from the decarboxylation of pyruvate and every mole formed results in the production of one mole of CO_2 and two moles of H_2 (equation 2.22, chapter 2). The formation of propionic acid does not result in the production of gas, instead two hydrogen atoms are used in the formation of propionic acid from elements equivalent to triose (equation 2.24, chapter 2). The CO_2 and H_2 produced in the formation of acetic or butyric acids may combine to form CH_4 (Hungate, 1966). Thus, if the fermentation of a particular feedstuff leads to the formation of fermentation acids with a high acetate:propionate ratio then the yield of gas will be greater than from a feedstuff which elicits a lower acetate:propionate ratio.

The amount of acidification gas produced during the fermentation is dependent upon the production of fermentation acids but is also related to the composition of the buffer used in the culture medium. With a bicarbonate buffer, for example, 1 mole of acidification gas will be produced for every mole of fermentation acid formed. However, the buffer used during gas production studies generally contain a phosphate buffer in addition to the bicarbonate buffer and thus, as neutralisation of the phosphate buffer does not result in the production of CO_2, the gas produced by acidification is less than 1 mole for every mole of acid formed (Beuvink & Spoelstra, 1992; see section 2.2.8).

Despite the above considerations, VFA ratios are generally not taken into account when comparing feedstuffs using *in vitro* gas production techniques. This may not be important if the feedstuffs are fermented to produce similar VFA molar proportions, but comparison of gas production profiles for feedstuffs which ferment to produce different VFA molar proportions may be quite misleading. The aim of this study was therefore to determine the relative contributions of acidification gas and fermentation gas to the overall gas pool resulting from the fermentation of feedstuffs with different NSP (fibre) and starch contents. This was with a view to establishing a method to standardise gas profiles from the fermentation of chemically diverse feedstuffs which yield different VFA molar ratios. Such standardisation should allow comparison of a wide range of different feeds to be ranked accordingly in terms of their nutritional value. The effect of VFA molar proportions on gas production profiles was investigated further using various grass and clover mixtures.

5.1.2 Materials and Methods

5.1.2.1 Measurement of volatile fatty acid (VFA) production throughout the incubation (Experiment 5.1.1)

Oatfeed, naked oats and soya hulls (0.2, 0.4. 0.6, 0.8 and 1.00 g x 3 replicates) were incubated with ovine rumen fluid for 60 h. Five bottles containing no substrate were included in the incubation to act as substrate negative controls, making the total number of bottles used 50. Gas production was recorded in these bottles at 3, 6, 9, 12, 15, 18, 21, 25, 29, 33, 37, 42, 47, 52 and 60 h after inoculation. Measurements of pH, VFA production and DM loss were made at the end of the incubation. For oatfeed and naked oats an additional 33 bottles (66 bottles in total), each containing 1.00 g substrate, were included in order to measure VFA production at ten time intervals (3 replicates per interval) throughout the incubation. After the first 3 h of the incubation, a needle was inserted through the stopper of each of these bottles to relieve the pressure during the incubation (i.e. gas accumulation was not measured in these bottles). In order to maintain an anaerobic environment these bottles were kept in a CO_2 chamber, at 39 °C, throughout the incubation. Bottles were harvested for

DM loss, NSP loss and VFA production measurements at 0, 3, 6, 9, 12, 15, 18, 21, 29, 37 and 47 h after inoculation.

The experiment was a factorial design consisting of 3 different substrates, 5 different substrate weights and 3 replicate bottles (3 x 5 x 3). The gas production profiles were fitted to the model of France *et al.* (1993) and compared using the parallel curve analysis function of MLP (Ross, 1987) (section 3.11). DM loss and VFA production profiles in the 1.00 g naked oats and oatfeed incubations were also analysed using parallel curve analysis (section 3.11). Values for the modelled gas production parameters, DM loss, VFA production and pH at the end of the incubation were analysed using analysis of variance in Genstat 5 (Lawes Agricultural Trust, 1993). Differences between treatments were calculated using the LSD (section 3.11).

5.1.2.2 Measurement of acidification gas production (Experiment 5.1.2)

Gas release from the culture medium (section 3.7) was measured after step-wise addition of either acetic, propionic or butyric acids, to each of a series of 25 serum bottles, containing 85 ml of culture medium, 4 ml reducing agent and 10 ml distilled water (to replace rumen digesta). Thirty minutes after the addition of 2 ml of one molar (i.e. 2 mmol) acetic, butyric or propionic acid, gas production was measured and a further 2 ml of acid added. After each gas reading, two bottles were removed for pH measurement. The procedure was repeated until 24 mmol of acid had been added. Bottles were kept at 39 °C throughout the experiment.

Both the cumulative gas production and the pH profiles were analysed using parallel curve analysis (section 3.11).

5.1.2.3 Measurement of volatile fatty acid (VFA) and gas production during incubation of perennial ryegrass, white clover and various grass:clover mixtures (Experiment 5.1.3)

Samples of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) were collected from IGER, Aberystwyth. The ryegrass was grown in an outdoor plot

whilst the clover was grown in a greenhouse under natural light conditions and watered daily. The chemical composition of the grass and clover samples is shown in Appendix 1. Immediately after cutting both the grass and clover samples were freeze dried for 48 h and ground through a 1 mm dry mesh screen.

Samples (3 x 1.00 g) of grass, clover or grass:clover (G:C) mixtures (80:20, 60:40, 40:60 and 20:80) were incubated with an ovine rumen microbial inoculum (section 3.2.1 and 3.4). Three bottles containing no substrate were included in the incubation to act as substrate negative controls, making the total number of bottles 21. Gas production was recorded at 0, 3, 6, 9, 12, 15, 18, 21, 24, 28, 32, 37, 46, 56, 72, 96 and 145 h. At the end of the incubation pH, DM loss and VFA production were determined (section 3.10 and 3.12.1).

The experiment was a factorial design consisting of 6 different grass : clover mixtures and 3 replicate bottles (6 x 3). The gas production profiles were fitted to the model of France *et al.* (1993) and compared using the parallel curve analysis function of MLP (Ross, 1987) (section 3.11). Values for the modelled gas production parameters, DM loss, VFA production and pH were analysed using analysis of variance in Genstat 5 (Lawes Agricultural Trust, 1993). Differences between treatments were calculated using the LSD (section 3.11).

5.1.3 Results

5.1.3.1 Measurement of volatile fatty acid (VFA) production throughout the incubation (Experiment 5.1.1)

5.1.3.1.1 Starch and non-starch polysaccharide (NSP) content and composition

The starch and NSP content and composition of the three experimental feedstuffs (naked oats, oatfeed and soya hulls) are shown in Table 5.1.1. Naked oats contained approximately 600 mg starch g^{-1} DM whereas oatfeed and soya hulls contained less at 110 and 10 mg g^{-1} respectively. By contrast, soya hulls and oatfeed contained greater levels of NSP than naked oats, the mean values being 559, 547 and 12 mg g^{-1} DM, respectively. The major NSP in soya hulls was glucose, accounting for *ca.* 270

Table 5.1.1 Starch and non-starch polysaccharide (NSP) content and composition of oatfeed, naked oats and soya hulls (mg g^{-1} DM).

Sample	Starch	NSP components							Total NSP
		Arabinose	Glucose	Galactose	Mannose	Rhamnose	Xylose	UAC	
Oatfeed	110	29.80	251.11	9.91	-	-	240.72	15.71	547.2
Naked oats	590	1.45	7.76	-	-	-	1.54	0.85	11.6
Soya hulls	10	39.04	270.83	20.20	43.03	4.85	58.50	122.82	559.2

NSP components (neutral sugars) were determined by gas chromatography from alditol acetate derivatives of acid hydrosylates of de-starched samples (section 3.12.3). Uronic acids (UAC) were determined by the colorimetric method of Scott (1979) (section 3.12.3). Total NSP was calculated by summing neutral sugars and uronic acids.

Starch was determined as described in section 3.12.4.

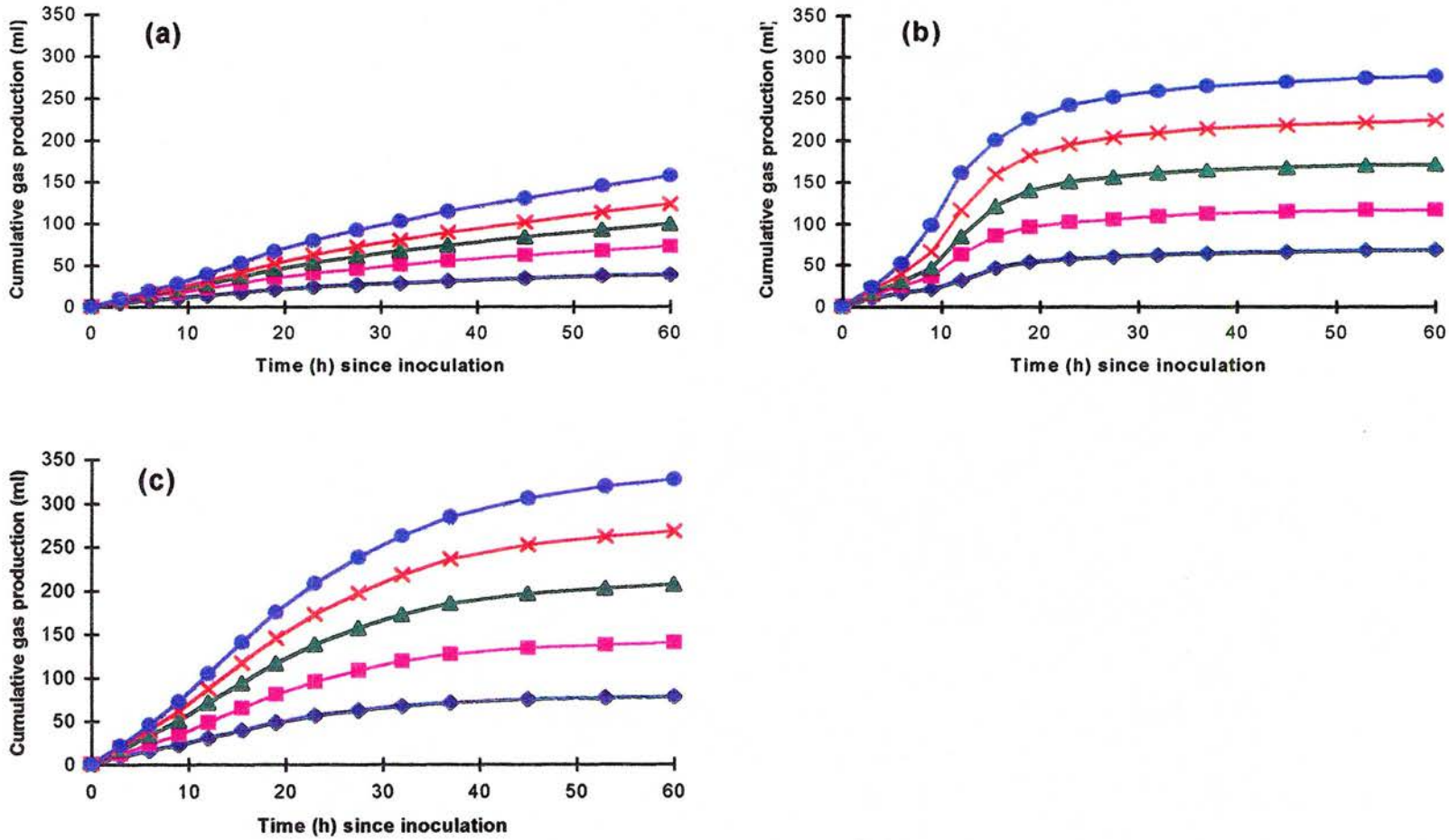
mg g^{-1} of the NSP, with the uronic acids being the next most abundant fraction, accounting for *ca.* 123 mg g^{-1} of the NSP. In oatfeed, glucose and xylose were most abundant , accounting for *ca.* 250 mg g^{-1} and 240 mg g^{-1} of the NSP, respectively. Glucose was also the most abundant NSP constituent of the naked oats, at approximately 8 mg g^{-1}, followed by xylose and arabinose at 1.5 and 1.4 mg g^{-1} of the NSP, respectively.

5.1.3.1.2 Gas production

The gas production profiles for each of the three feedstuffs are shown in Figure 5.1.1. Parallel curve analysis of the gas production profiles from 1.0 g naked oats, oatfeed and soya hulls indicated significant differences between the gas production profiles for the different feedstuffs ($p < 0.001$; Appendix 5.1.1). Incubation of smaller sample sizes (0.2, 0.4, 0.6 and 0.8 g) resulted in similar gas production profiles to those obtained during incubation of 1 g of substrate, with proportionately smaller quantities of gas produced (Figures 5.1.1 and 5.1.2). Figure 5.1.2 shows the relationship between gas production and the amount of substrate incubated for all three substrates. The different gradients of the lines (yields of gas per substrate weight) in Figure 5.1.2 suggest that the different feeds result in the production of VFA with different molar percentages and thus different yields of gas. If the fermentation had resulted in a similar VFA profile for all feeds, the volume of gas produced for each feed may have been different, but the gradient of the lines should have been the same.

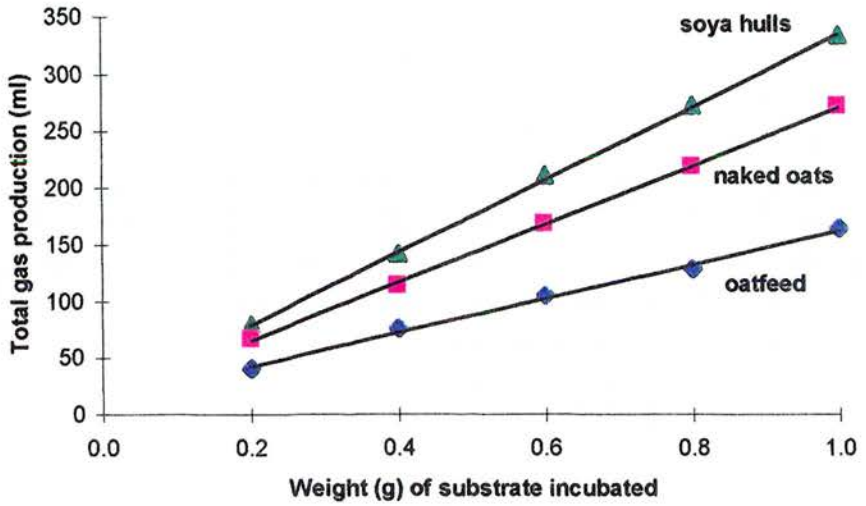
Fitted parameters and derived quantities for the gas production profiles are shown in Table 5.1.2. The rates of gas production, b and c, ranged from 0.0543 h^{-1} (for 0.2 g oatfeed) to 0.2769 h^{-1} (for 0.6 g naked oats) and from -1.0939 $h^{-0.5}$ (for 0.6 g naked oats) to -0.1084 $h^{-0.5}$ (for 0.2 g oatfeed), respectively. The mean value of b for all feeds was 0.1374 ± 0.02245 h^{-1}, whilst the mean value of c for all feeds was -0.4864 ± 0.09056 $h^{-0.5}$. Least gas was produced during the incubation of 0.2 g oatfeed (40.5 ml), whilst most gas was produced during incubation of 1.0 g soya hulls (333.2 ml). The mean total gas production across all feedstuffs and substrate weights was 159.3 ± 22.30 ml. The lag time, L_T, ranged from 1.0 h for 0.2 g oatfeed to 3.9 h for 0.6 g

Figure 5.1.1 Cumulative gas production for 0.2 (-◆-), 0.4 (-■-), 0.6 (-▲-), 0.8 (-X-) and 1.0 g (-●-) of **(a)** oatfeed, **(b)** naked oats and **(c)** soya hulls during 60 h incubation with a rumen microbial inoculum.



Fermentations were conducted in 160 ml serum bottles with 89 ml of culture medium and 10 ml of rumen microbial inoculum. Each value represents the mean of three bottles, in all cases SE were less than 5 % of the values shown in the figures.

Figure 5.1.2 Total gas production after 60 h incubation of 0.2, 0.4, 0.6, 0.8 and 1.0 g oatfeed, naked oats and soya hulls with a rumen microbial inoculum.



Fermentations were conducted in 160 ml serum bottles with 89 ml culture medium and 10 ml of rumen microbial inoculum. Each value is the mean of three bottles, in all cases SE were less than 5 % of the values shown in the figure. There was a good correlation between the total gas production and the weight of substrate incubated, with an R^2 value of greater than 0.99 for all feeds. The equation of the lines were as follows; oatfeed $y = 150.15x + 12.4$, naked oats $y = 256.98x + 14.0$, and for soya hulls $y = 320x + 15.15$. The different gradients of the lines may indicate different molar ratios of VFA.

Table 5.1.2. The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for naked oats (NO), oatfeed (OF) and soya hulls (SH) during incubation with a rumen microbial inoculum

Gas production parameter	*Substrate*	*Weight (g)*					
		0.2	0.4	0.6	0.8	1.0	s.e.d
b (h^{-1})	Oatfeed	0.0543^{a}	0.0585^{a}	0.0605^{a}	0.0619^{a}	0.0612^{a}	
	Soya hulls	0.1087^{b}	0.1088^{b}	0.1013^{b}	$0.0963^{b,c}$	0.0929^{c}	
	Naked oats	0.2064^{d}	0.2768^{e}	0.2769^{e}	0.2634^{f}	0.2331^{g}	0.0061
c ($h^{-0.5}$)	Oatfeed	-0.1084^{a}	-0.1682^{b}	-0.1927^{b}	-0.2132^{b}	-0.2071^{b}	
	Soya hulls	$-0.3492^{c,d}$	-0.3762^{c}	$-0.3399^{c,d}$	$-0.3255^{c,d}$	-0.3090^{d}	
	Naked oats	-0.7731^{e}	-1.0613^{f}	-1.0939^{f}	-0.9956^{g}	-0.7834^{e}	0.0266
A (ml)	Oatfeed	40.46^{a}	75.32^{b}	104.23^{d}	128.38^{f}	164.07^{h}	
	Soya hulls	78.86^{b}	141.06^{g}	210.27^{i}	272.29^{k}	333.24^{l}	
	Naked oats	67.37^{c}	114.01^{e}	168.59^{h}	219.27^{j}	271.72^{k}	2.4210
B	Oatfeed	$34.95^{a,b}$	60.78^{c}	82.09^{e}	99.13^{f}	128.18^{g}	
	Soya hulls	52.38^{d}	92.40^{f}	144.01^{h}	190.16^{i}	239.29^{j}	
	Naked oats	27.67^{a}	36.47^{b}	52.18^{d}	78.28^{e}	130.31^{g}	3.6590
L_T (h)	Oatfeed	1.00^{a}	2.07^{b}	2.54^{c}	2.96^{e}	2.86^{e}	
	Soya hulls	$2.58^{c,d}$	2.99^{e}	$2.81^{d,e}$	2.85^{e}	$2.76^{c,e}$	
	Naked oats	3.50^{f}	$3.69^{f,g}$	3.90^{g}	3.57^{f}	$2.81^{d,e}$	0.1213
t_{50} (h)	Oatfeed	18.73^{a}	21.51^{b}	22.67^{c}	23.81^{d}	23.78^{d}	
	Soya hulls	15.09^{e}	16.56^{f}	16.88^{f}	17.68^{g}	18.00^{g}	
	Naked oats	12.01^{h}	$11.28^{i,j}$	11.88^{h}	$11.59^{h,i}$	10.89^{j}	0.2864
t_{95} (h)	Oatfeed	69.13^{a}	71.90^{b}	$72.71^{b,c}$	73.76^{c}	73.99^{c}	
	Soya hulls	45.45^{d}	47.49^{e}	49.38^{f}	51.69^{g}	52.87^{g}	
	Naked oats	31.05^{h}	26.49^{i}	27.18^{i}	27.17^{i}	27.22^{i}	0.6500

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 5.1.1. Values for each parameter not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables are shown in Appendix 5.1.2.1 - 5.1.2.7.

naked oats. The mean value of L_T for all feeds was 2.86 ± 0.183 h. The time taken to produce 50 % of the total gas production, t_{50}, ranged from 10.89 h for 1.0 g naked oats to 23.78 h for 1.0 g oatfeed. The mean value of t_{50} for all feeds was 16.82 ± 1.202 h. The time taken to produce 95 % of the total gas production, t_{95}, was quickest for 0.4 g naked oats (26.49 h) and slowest for 1.0 g oatfeed (73.99 h). The mean value for t_{95} across all feeds was 49.83 ± 4.885 h.

5.1.3.1.3 Dry matter loss

Parallel curve analysis of the DM loss profiles for 1.0 g oatfeed and naked oats during the incubation, indicated a significant difference in DM loss between the samples. With a significantly greater proportion of the naked oat DM disappearing compared to oatfeed ($p < 0.001$; Appendix 5.1.3.1).

DM loss (normalised across substrate weights to mg g^{-1}) from the different quantities of oatfeed, soya hulls and naked oats after 60 h incubation are shown in Table 5.1.3. Analysis of variance showed that the greatest DM loss occurred after incubation of naked oats (with a mean value for all substrate weights of 966.2 ± 3.27 mg g^{-1}), with significantly less DM being degraded from soya hulls (with a mean value for all substrate weights of 869.3 ± 6.27 mg g^{-1}) and least from oatfeed (with a mean value for all substrate weights of 371.3 ± 1.80 mg g^{-1}). The weight of substrate incubated generally had no significant effect on the DM loss from the substrate indicating that these batch fermentations were limited by the amount of available substrate and not by any other factors which were presumably all present in excess of their requirements.

Table 5.1.3 Dry matter (DM) loss (mg g^{-1}) from 0.2, 0.4, 0.6, 0.8 and 1.0 g oatfeed, soya hulls and naked oats after 60 h incubation with a rumen microbial inoculum.

Substrate	Weight (g)					
	0.2	0.4	0.6	0.8	1.0	sed
Oatfeed	372.7^{a}	371.1^{a}	364.5^{a}	374.9^{a}	373.3^{a}	
Soya hulls	893.3^{b}	870.1^{c}	860.5^{c}	863.4^{c}	859.4^{c}	
Naked oats	$962.8^{d,e}$	$966.3^{d,e}$	$965.1^{d,e}$	958.6^{d}	978.2^{e}	8.25

Values not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance table for the above data is shown in Appendix 5.1.3.2.

5.1.3.1.4 Loss of non-starch polysaccharides (NSP)

Loss of NSP ranged from 789 mg g^{-1} NSP (oatfeed) to > 990 mg g^{-1} (naked oats). Extensive degradation of the naked oat samples during incubation meant there was insufficient residue for NSP analysis at several incubation times, therefore, loss of NSP throughout the incubation from oatfeed, only, is shown in Table 5.1.4. Of the oatfeed NSP constituents, glucose was degraded to the greatest extent (848 mg g^{-1} glucose), followed by galactose (780.8 mg g^{-1} galactose), arabinose (753.4 mg g^{-1} arabinose), xylose (747.7 mg g^{-1} xylose) and uronic acids (535.7 mg g^{-1} uronic acids).

5.1.3.1.5 Volatile fatty acid (VFA) production

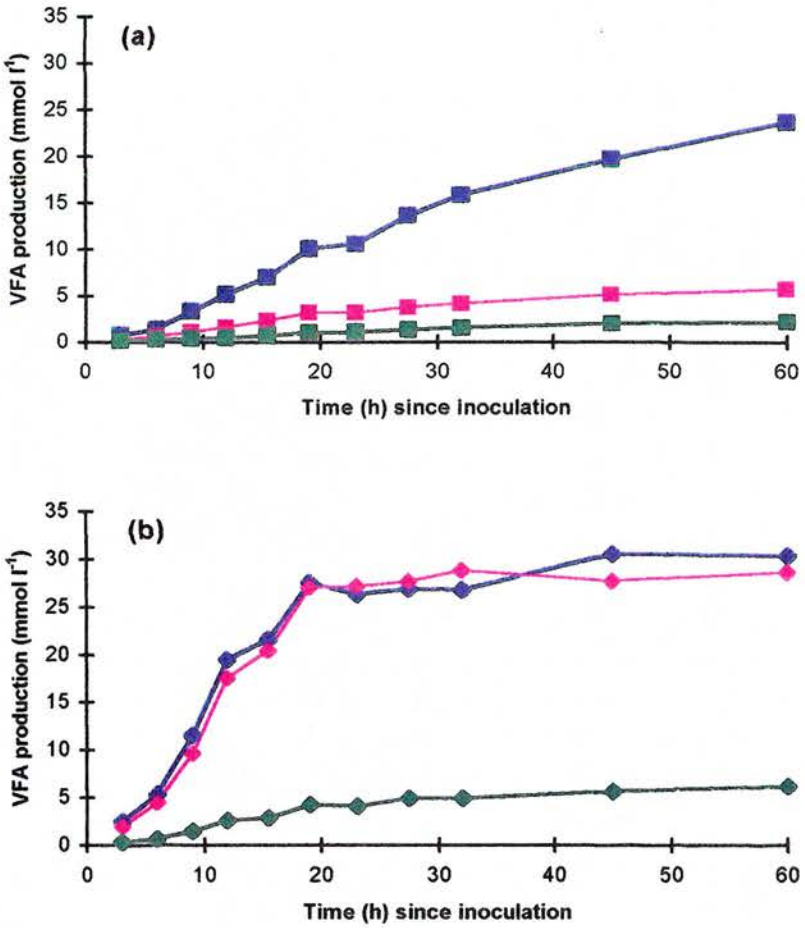
Parallel curve analysis of the VFA production during 0 - 45 h incubation, for oatfeed and naked oats indicated a significantly greater production of acetic, propionic and butyric acids during incubation of naked oats compared to oatfeed ($p < 0.001$; Figure 5.1.3; Appendix 5.1.4).

Analysis of variance of the VFA present after 60 h incubation of 1.0 g oatfeed, naked oats and soya hulls indicated significant differences between feeds (Table 5.1.5). Total VFA production was greatest after incubation of both naked oats and soya hulls (66.51 and 72.34 mmol l^{-1}, respectively), whilst incubation of oatfeed produced significantly less total VFA (31.88 mmol l^{-1}) ($p < 0.01$). The molar percentages of acetate, propionate and butyrate also differed significantly between the feeds. Oatfeed

Table 5.1.4 Loss of non-starch polysaccharide components from oatfeed (mg g^{-1} NSP or NSP constituents) throughout a 60 h incubation with a rumen microbial inoculum.

Time (h)	Loss of NSP components (mg g^{-1} NSP constituent)							Total NSP loss (mg g^{-1} NSP)
	Arabinose	Glucose	Galactose	Mannose	Rhamnose	Xylose	UAC	
0	65.4	202.9	-	-	-	48.1	512.1	122.2
3	7.1	-	-	-	-	41.7	436.9	-
6	562.1	151.4	70.7	-	-	68.7	575.2	148.1
9	73.3	286.8	97.0	-	-	173.3	635.0	241.2
12	221.1	348.3	119.2	-	-	238.6	605.7	296.4
16	11.7	88.8	-	-	-	114.9	425.5	95.6
20	201.7	167.8	181.8	-	-	161.7	470.1	175.9
24	241.3	226.0	216.2	-	-	199.7	781.5	231.0
28	224.8	217.8	177.8	-	-	159.6	747.1	207.0
32	233.9	251.5	229.3	-	-	216.9	431.8	240.1
45	507.0	540.0	450.5	-	-	474.1	803.2	515.1
60	753.4	848.0	780.8	-	-	747.7	535.7	788.6

Figure 5.1.3 VFA production; acetate (—), propionate (—) and butyrate (—) (mmol l^{-1}) during incubation of **(a)** oatfeed and **(b)** naked oats with a rumen microbial inoculum.



Fermentations were conducted in 160 ml serum bottles with 89 ml culture medium and 10 ml of rumen microbial inoculum. Three bottles were harvested for VFA analysis at 0, 3, 6, 9, 12, 15, 18, 21, 29, 37, 47 and 60 h after inoculation. Each value is the mean value for three bottles, in all cases SE were less than 5 % of the values shown in the figure.

resulted in the greatest percentage of acetate, whilst incubation of naked oats produced the lowest percentage of acetate. The highest percentages of propionate and butyrate were produced after incubation of the naked oats, with the lowest percentages produced during incubation of oatfeed. Like total VFA production, naked oats and soya hulls produced similar percentages of valerate whilst the lowest percentage of valerate was produced during incubation of oatfeed.

Table 5.1.5 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced from 1.0 g oatfeed, naked oats and soya hulls after 60 h incubation with a rumen microbial inoculum.

	Feed				
	Naked oats	Oatfeed	Soya hulls	s.e.d	sig.
Total VFA (mmol l^{-1})	66.51^{a}	31.88^{b}	72.34^{a}	2.440	**
Acetate (molar %)	45.59^{a}	74.15^{b}	70.34^{c}	0.415	**
Propionate (molar %)	42.90^{a}	17.54^{b}	20.00^{c}	0.367	**
Butyrate (molar %)	9.19^{a}	6.73^{b}	7.40^{c}	0.149	*
Valerate (molar %)	2.31^{a}	1.56^{b}	2.25^{a}	0.047	**

Values in rows not bearing the same superscripts differ significantly (**$p < 0.01$; *$p < 0.05$). The analysis of variance tables are shown in Appendix 5.1.5.1 - 5.1.5.5.

5.1.3.1.6 Changes in batch culture pH

At the end of the incubation the pH of the culture medium was significantly lower in bottles containing naked oats (6.5 ± 0.03 pH units) compared to those containing oatfeed (6.6 ± 0.01 pH units) ($p < 0.05$; Appendix 5.1.6), although this small difference is unlikely to be biologically significant. The small decline in the pH of the culture medium observed throughout the incubation, indicated the efficiency of the buffer at this level of VFA production.

5.1.3.2 Measurement of acidification gas production (Experiment 5.1.2)

5.1.3.2.1 Gas production

The gas production profiles resulting from the addition of acetic, propionic and butyric acids to the culture medium are shown in Figure 5.1.4. All three profiles were similar, with gas production increasing linearly until approximately 12 mmoles of acid had been added to the culture medium. Parallel curve analysis indicated no significant differences between the gas production profiles (Appendix 5.1.7). The average volume of gas produced from the culture medium upon the addition of 24 mmoles of acetic, propionic or butyric acid was 236 ± 0.81 ml.

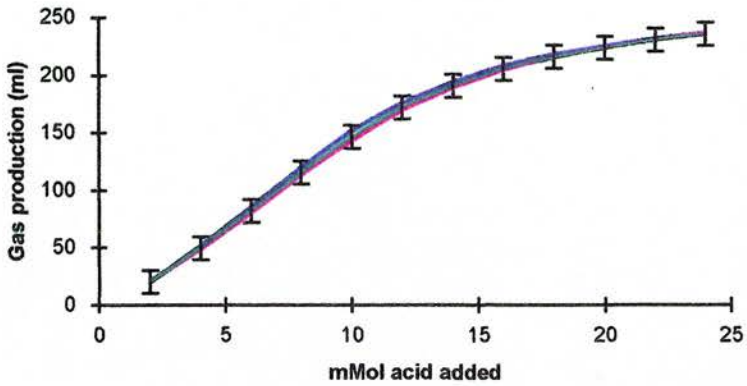
In order to determine the moles of gas released from the medium upon the addition of one mole of acid, linear regression analysis was carried out on the data from the linear part of the curve (up to the addition of 12 mmol). The mean value for all acids was 0.62 ± 0.015 moles of gas per mole of acid added. Assuming the ideal gas law; at 39 °C and at 1 atmosphere, 1 mole of gas has a theoretical volume of 25.6 litres (section 2.2.3.4). This was used to determine the gas production in ml from the medium for each mole of acid added, i.e.:

1 mole acid → 0.62 moles gas → 15.9 litres

5.1.3.2.2 Changes in batch culture pH

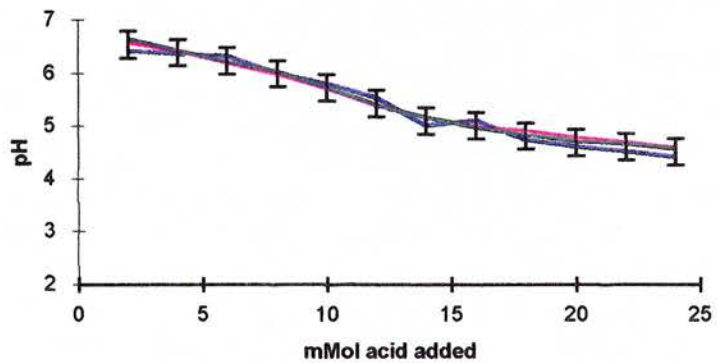
The decline in culture medium pH with the addition of each acid is shown in Figure 5.1.5. Parallel curve analysis indicated no significant differences between acids in the decline in pH with increasing acid added (Appendix 5.1.8). The pH ranged from 6.72 ± 0.015 to 4.47 ± 0.09, 4.54 ± 0.010 and 4.57 ± 0.003 after the addition of 24 mmoles of acetic, butyric or propionic acid. This represented a decline in pH of 2.25, 2.18 and 2.15 pH units for the addition of acetic, butyric and propionic acids, respectively.

Figure 5.1.4 Cumulative gas production following stepwise addition of acetic (—), propionic (—) or butyric (—) acid to the culture medium (acidification gas).



Fermentations were conducted in 160 ml serum bottles with 89 ml culture medium and 10 ml distilled water (no microbial inoculum was used). Each line is the mean gas production profile of three bottles, in all cases SE were less than 5 % of the values shown in the figure. I represents the addition of acid.

Figure 5.1.5 Decline in culture medium pH with stepwise addition of acetic (—), propionic (—) or butyric (—) acid.



Fermentations were conducted in 160 ml serum bottles with 89 ml culture medium and 10 ml distilled water (no microbial inoculum was used). Each line represents the decline in pH for three bottles. The pH of the culture fluid was measured 30 min after each addition of 2 ml of 1 M acetic, propionic or butyric acid. I represents the addition of acid.

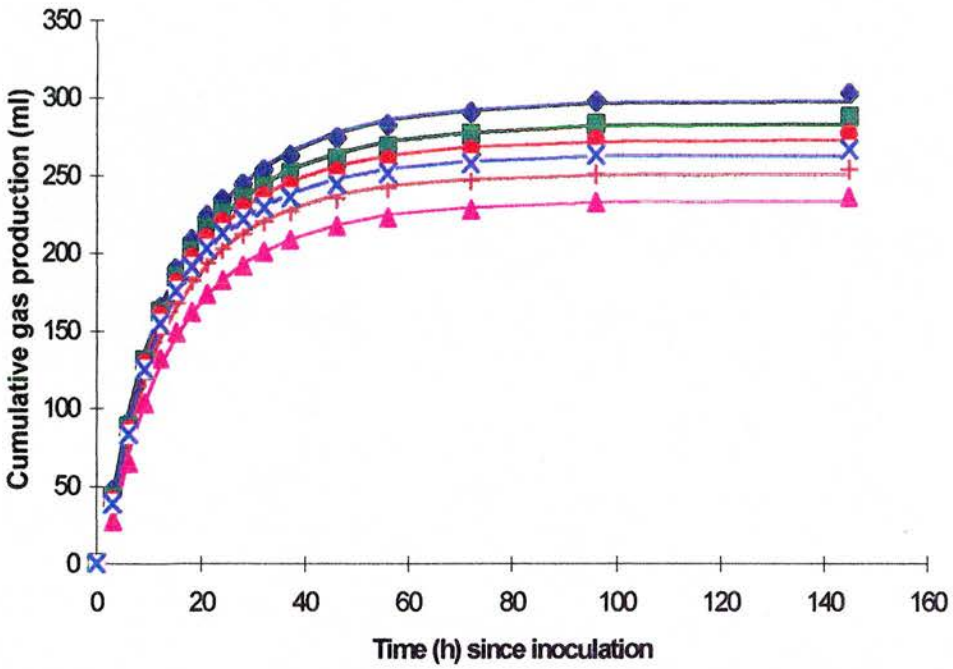
5.1.3.3 Measurement of volatile fatty acid (VFA) and gas production during incubation of perennial ryegrass, white clover and various grass:clover mixtures (Experiment 5.1.3)

5.1.3.3.1 Gas production

The gas production profiles for grass, clover and the different G:C mixtures are shown in Figure 5.1.6. Parallel curve analysis of the gas production profiles indicated significant differences in the volume of gas produced; the largest volume of gas was produced by 100 % grass, followed by 80:20 G:C, 60:40 G:C, 40:60 G:C, 20:80 G:C, with least gas being produced during incubation of 100 % clover ($p < 0.001$; Appendix 5.1.9). Parallel curve analysis indicated significant differences in the rate of gas production between 100 % grass and both 20:80 G:C and 40:60 G:C ($p < 0.05$; Appendix 5.1.9). No significant differences were observed between the remaining data sets.

Fitted parameters and derived quantities for the gas production profiles are shown in Table 5.1.6. The rate of gas production, b and c, were similar for all grass:clover mixtures ranging from 0.0327 h^{-1} for the 80:20 and 60:40 grass :clover mixtures to 0.0383 h^{-1} for the 20:80 grass:clover mixture and from 0.1779 $h^{-0.5}$ for 100 % grass to 0.2451 $h^{-0.5}$ for the 60:40 grass:clover mixture, for b and c respectively. The mean value for b was 0.0352 ± 0.00101 h^{-1}, and the mean value for c was 0.2216 ± 0.01043 $h^{-0.5}$. The total volume of gas produced was greatest after incubation of 100 % grass (298 ml) with progressively smaller quantities of gas produced as the percentage of grass in the mixtures decreased, with 100 % clover producing least gas (233 ml). The mean value for the total gas production for all mixtures was 267 ± 9.45 ml. The lag time (L_T) was shortest during incubation of 100 % grass (1.42 h) and gradually increased as the percentage of clover in the mixture increased with 100 % clover showing the greatest lag time (1.94 h). The mean lag time for all grass:clover mixtures was 1.71 ± 0.072 h. The time taken to produce 50 % of the total gas production, t_{50}, ranged from 9.5 h for the 40:60 G:C mixture to 10.5 h for 100 % clover, with a mean value for all samples of 9.9 ± 0.17 h. The time taken to produce

Figure 5.1.6 Cumulative gas production profiles for grass (◆), clover (▲) and various grass clover mixtures (80:20 ■; 60:40 ●; 40:60 ×; 20:80 +) during incubation with a rumen microbial inoculum.



Fermentations were conducted in 160 ml serum bottles with 89 ml of culture medium and 10 ml of rumen microbial inoculum. Each value represents the mean of three bottles, whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure.

95 % of the total gas production, t_{95}, ranged from 47.5 h for 20:80 G:C to 52.7 h for 100 % grass with a mean value for all samples of 50.5 ± 0.78 h.

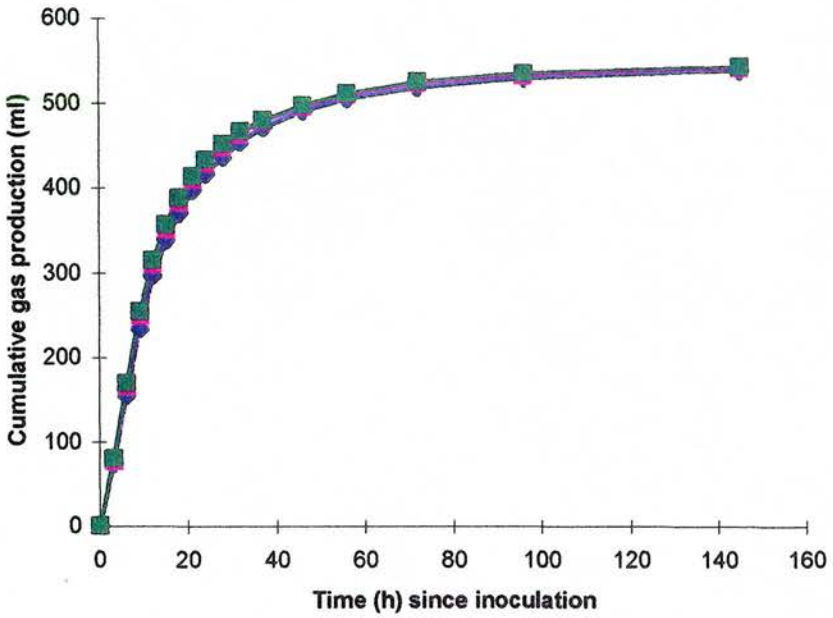
Table 5.1.6 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for 100 % grass (100:0 G:C), 100 % clover (0:100 G:C) and various grass clover mixtures (G:C) during incubation with a rumen microbial inoculum

Gas production parameters	Grass : Clover mixtures						
	100:0	80:20	60:40	40:60	20:80	0:100	sed
b (h^{-1})	0.0375	0.0327	0.0327	0.0337	0.0383	0.0363	0.0031
c ($h^{-0.5}$)	0.1779^{a}	0.2296^{b}	0.2451^{b}	0.2440^{b}	0.2262^{b}	$0.2069^{a,b}$	0.0204
A (ml)	297.9^{a}	$282.8^{a,b}$	272.5^{b}	262.8^{c}	250.3^{c}	233.1^{d}	7.05
B	$388.8^{a,b}$	400.9^{a}	397.3^{a}	$384.1^{a,b}$	364.5^{b}	333.8^{c}	11.18
L_T (h)	1.42^{a}	1.64^{b}	1.71^{b}	1.73^{b}	1.83^{c}	1.94^{d}	0.0399
t50 (h)	10.34^{a}	9.84^{b}	9.57^{b}	9.51^{b}	9.62^{b}	10.52^{a}	0.1745
t95 (h)	52.70^{a}	51.87^{a}	50.17^{b}	$49.41^{b,c}$	47.46^{c}	$51.52^{a,b}$	1.0530

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 5.1.6. Values in rows not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables are shown in Appendix 5.1.10.1 - 5.1.10.7.

In order to see if the gas production profiles were additive, corresponding grass:clover mixtures were added together to give three gas production profiles for a 1:1 grass:clover mixture (i.e. mixture 1 = 100:0 G:C + 0:100 G:C; 2 = 80:20 G:C + 20:80 G:C and 3 = 60:40 G:C + 40:60 G:C). The resulting gas production profiles were all similar (Figure 5.1.7). Fitted parameters and derived quantities for these additive gas production profiles are shown in Table 5.1.7. The rates of gas production, b and c, were similar for all three curves, parallel curve analysis indicating no significant differences between the rates of gas production (Appendix 5.1.11). Hence, values for t_{50} and t_{95} were also similar between the curves. Values for t_{50} ranged from 9.5 to 10.4 h with a mean value of 9.9 ± 0.47 h, whilst t_{95} ranged from

Figure 5.1.7 Additive gas production profiles for 100 % clover + 100 % grass (◆), 80:20 G:C + 20:80 G:C (▲) and 60:40 G:C + 40:60 G:C (■) during incubation with a rumen microbial inoculum.



Fermentations were conducted in 160 ml serum bottles with 89 ml of culture medium and 10 ml of rumen microbial inoculum. Each value represents the mean of three bottles plus the mean value from three complimentary bottles (i.e. 100 % grass + 100 % clover, 80:20 grass:clover (G:C) + 20:80 G:C and 60:40 G:C plus 40: 60 G:C), whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure.

49.6 to 51.1 h with a mean value of 50.5 ± 1.42 h. The lag time (L_T) was approximately 1.7 h for the different curves with a mean value of 1.70 ± 0.04 h. Although the total volume of gas produced (A) was very similar for all three curves, ranging from 531 to 535 ml with a mean value of 533.1 ± 2.15 ml, parallel curve analysis indicated significant differences between the curves. The volume of gas produced from 100 % grass plus 100 % clover being significantly less than that produced from both 80:20 plus 20:80 G:C ($p < 0.01$) and 60:40 plus 40:60 G:C ($p < 0.001$; Appendix 5.1.11).

Table 5.1.7 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for 1:1 grass : clover (G:C) mixtures, calculated from 100 % grass plus 100 % clover, 80:20 G:C plus 20:80 G:C and 60:40 G:C plus 40:60 G:C, obtained during incubation with a rumen microbial inoculum

	Gas production parameters					
Substrate	b (h^{-1})	c ($h^{-0.5}$)	A (ml)	L_T (h)	t_{50} (h)	t_{95} (h)
grass + clover	0.0370	0.1902	531.0	1.66	10.43	52.15
80:20 + 20:80 G:C	0.0353	0.2278	533.0	1.73	9.73	49.62
60:40 + 40:60 G:C	0.0331	0.2444	535.3	1.72	9.54	49.77

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 5.1.7. No significant differences were detected between the different substrates (Appendix 5.1.11).

The additive nature of the gas production profiles was investigated further by predicting the gas production profiles of the different G:C mixtures from the 100 % grass and 100 % clover gas production profiles only. For example, gas production from the 80:20 G:C mixture was predicted by adding 80 % of the gas production profile obtained from incubation of 100 % grass with 20 % of that obtained during incubation of 100 % clover. The predicted gas production profile correlated well with the actual gas production profile obtained ($R^2 = 0.999$; Figure 5.1.8.a). Prediction of the gas production profiles for the remaining G:C mixtures was also investigated and

the profiles correlated well with the actual volumes of gas measured during the experiment with all R^2 values equal to 0.999 (Figure 5.1.8).

5.1.3.3.2 Dry matter loss

The extent of DM loss varied for the different grass / clover mixtures (Table 5.1.8). The greatest extent of DM loss was for 100 % grass (905.7 mg g^{-1}) and the 60:40 G:C mixture (898.1mg g^{-1}), whilst 100 % clover (839.0 mg g^{-1}) and 20:80 G:C mixture (846.4 mg g^{-1}) were degraded to the least extent.

Table 5.1.8 Dry matter loss (mg g^{-1}) of grass, clover and various grass / clover mixtures during incubation with a rumen microbial inoculum.

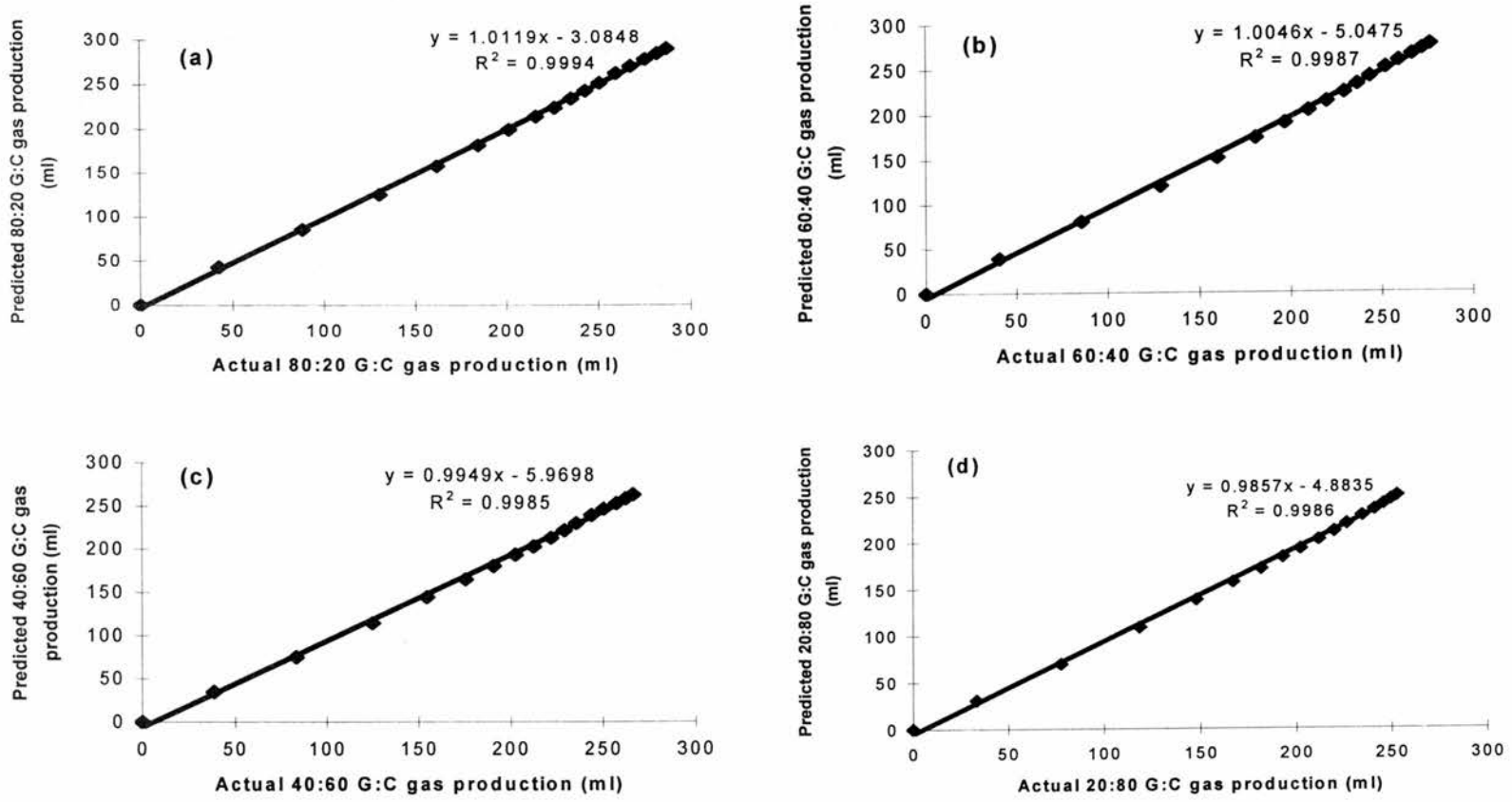
grass : clover mixtures					
100:0	80:20	60:40	40:60	20:80	0:100
905.7^{a}	$855.7^{b,c}$	$898.1^{a,c}$	875.2^{c}	846.4^{b}	839.0^{b}

Values not bearing the same superscript differ significantly ($p < 0.05$). sed was 13.04. The analysis of variance table is shown in Appendix 5.1.12.

5.1.3.3.3 Volatile fatty acid (VFA) production

VFA production after incubation of the grass, clover and G:C mixtures are shown in Table 5.1.9. Total VFA production at the end of the incubation was similar for 100 % grass, 100 % clover and all G:C mixtures, with the exception of 40:60 G:C which produced significantly more total VFA than 20:80 G:C ($p < 0.05$). There were also significant differences in the molar percentages of the VFA produced by grass, clover and the various G:C mixtures (Table 5.1.9). The percentage of Ac tended to increase as the proportion of clover in the mixture increased, whilst the percentage of propionate tended to decrease as the proportion of clover in the mixture increased. The percentage of Bu was similar between all mixtures, with the exception of 100 % grass which produced a significantly lower percentage of Bu than 20:80 G:C ($p < 0.05$). A significantly higher percentage of Val was produced during incubation of 100 % clover and 20:80 G:C compared to all other mixtures ($p < 0.05$). There were also significant differences between 40:60 G:C and both 100 % grass and 80:20 G:C

Figure 5.1.8 Predicted gas production for different grass : clover (G:C) mixtures (calculated from 100 % grass and 100 % clover gas production profiles) versus actual gas production for **(a)** 80:20 G:C, **(b)** 60:40 G:C, **(c)** 40:60 G:C and **(d)** 20:80 G:C.



Gas production during incubation of different grass:clover mixtures (actual gas production) was predicted from the gas production profiles from 100 % grass and 100 % clover.

($p < 0.05$), whilst 60:40 G:C produced a significantly higher percentage of Val than 100 % grass.

Table 5.1.9 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate from grass, clover and various grass : clover mixtures after incubation with a rumen microbial inoculum.

	grass : clover mixtures						
	100:0	80:20	60:40	40:60	20:80	0:100	sed
Total VFA (mmol l^{-1})	$72.49^{a,b}$	$71.37^{a,b}$	$73.00^{a,b}$	74.22^{b}	66.29^{a}	$67.65^{a,b}$	3.198
Acetate (molar %)	58.31^{a}	57.98^{a}	58.89^{a}	$59.42^{a,c}$	$60.47^{b,c}$	61.70^{b}	0.677
Propionate (molar %)	31.65^{a}	31.40^{a}	30.03^{c}	29.23^{c}	26.37^{b}	25.03^{b}	0.603
Butyrate (molar %)	7.26^{a}	$7.61^{a,b}$	$7.48^{a,b}$	$7.53^{a,b}$	7.81^{b}	$7.48^{a,b}$	0.231
Valerate (molar %)	2.78^{a}	$3.01^{a,c}$	$3.61^{c,d}$	3.83^{d}	5.35^{b}	5.79^{b}	0.286

Values in rows not bearing the same superscripts differ significantly ($p < 0.05$). The analysis of variance tables are shown in Appendix 5.1.13.1 - 5.1.13.5.

5.1.3.3.4 Changes in batch culture pH

The pH at the end of the incubation was seen to increase as the proportion of clover in the mixture increased. A significantly lower pH was recorded in bottles containing 100 % grass (6.3) and 80:20 G:C (6.2) compared to bottles containing 60:40 G:C (6.4) ($p < 0.05$). Whilst the pH after incubation of 100 % clover (6.7), 20:80 G:C (6.7) and 40:60 G:C (6.6) was significantly higher than that in bottles containing 60:40 G:C ($p < 0.05$; Appendix 5.1.14).

5.1.4 Discussion

The changes in fermentation pattern which occur when different diets are fed are often used in ruminant nutrition as a way of manipulating animal production. The ratio of VFA produced during digestion in the rumen depends upon the nature of the

diet fed; high concentrate (starch rich) diets enhance propionic acid production at the expense of acetic and butyric acids, whilst high fibre diets tend to favour the production of acetic and butyric acids (McDonald *et al.*, 1995). As the quantity of gas produced during the formation of these acids varies (section 5.1.1) it is important to consider the VFA molar proportions produced, especially when comparing gas production profiles from widely different substrates, such as comparing a forage with a cereal grain.

From the stoichiometry described by Wolin (1960) and Hungate (1966) it should be possible to predict how much fermentation gas will be produced during the fermentation of a substrate from the quantity of VFA which is produced. This stoichiometry was successfully used by Orskov *et al.*, (1968) and Whitelaw *et al.*, (1970) to estimate the production of VFA and total fermented energy from measurements of methane production and the molar proportions of acetic, propionic and butyric acids. The ability to predict how much gas will be produced during the fermentation of a substrate from the VFA production would enhance our understanding of gas production profiles and allow us to compare different substrates on a common basis.

The amount of gas expected to be produced during fermentation of the feeds was calculated from the amount and type of VFA produced and their respective proportions. Determination of the expected gas production involved calculating the CO_2 and H_2 produced during the fermentation (fermentation gas) using the stoichiometry described by Hungate (1966) and the CO_2 released from the buffer (acidification gas; section 2.2.8.3). A summary of fermentation and acidification gas production from the formation of each VFA is shown in Table 5.1.10.

Table 5.1.10 Fermentation and acidification gas production from the fermentation of 1 mole of glucose to acetic, butyric or propionic acid.

VFA (moles)	[1]Fermentation gas production (moles)	[2]Acidification gas production (moles)	Total gas production (moles)
2 Ac	2 CO_2 , 4 H	(2 x 0.62)	3.24 CO_2 , 4 H
1 Bu	2 CO_2 , 2 H	(1 x 0.62)	2.62 CO_2 , 2 H
2 Pr	-2 H	(2 x 0.62)	1.24 CO_2 , -2 H

[1]Fermentation gas production refers to the gas produced when 1 mole of glucose is fermented to give either acetic, butyric or propionic acid. [2]Acidification gas refers to the volume of CO_2 released from the medium upon the addition of 1 mole of VFA (1 mole acid did not release 1 mole CO_2 from the medium due to the presence of a phosphate buffer in addition to the bicarbonate buffer).

The addition of 1 mole of acid, to the culture medium, would be expected to produce 1 mole of gas, however, the buffer contained a phosphate as well as a bicarbonate buffer and hence less than the expected 1 mole of gas was released upon the addition of 1 mole of acid. The rate of acidification gas production remained constant over a slight change in pH, therefore pH decline, under the conditions tested, did not alter the rate of acidification gas production. From Table 5.1.10, the production of 1 mole of acetic acid, in this *in vitro* system, results in the production of 1.62 moles of CO_2 and 2 moles of H; 1 mole of butyric acid results in the production of 2.62 moles of CO_2 and 2 moles of H, and 1 mole of propionic acid results in the production of 0.62 moles of CO_2 and uses 1 mole of H.

In gas production experiments with the pressure transducer technique, VFA production is generally between 0 - 9 mmol. Therefore, given that 9 mmol acid will produce 5.58 mmol gas (each mmol acid will produce 0.62 mmol acidification gas) up to 143 ml of the total gas production during the incubation of a feed may be acidification gas (Figure 5.1.4). Moreover, as the gas production from the culture medium increased linearly from 0 - 12 mmol acid added, the acidification gas production should be constant throughout gas production experiments. The acidification gas appeared to be approaching an asymptote of gas production at 24

mmol acid, hence the maximum volume of acidification gas which could be expected during any fermentation with this culture medium would be approximately 240 ml. As the buffer becomes exhausted, the production of more fermentation acid will not result in the production of additional acidification gas.

Using the above information (acidification gas as detailed in section 5.1.3.2 and the stoichiometry described by Hungate (1966); Table 5.1.10), the expected (theoretical) gas production was calculated for experiment 5.1.1 (1.0 g naked oats at 60 h incubation);

Experimental data (section 5.1.3.1.5)

Acetic acid = 30.31 mmol l^{-1} → 3.03 mmol $bottle^{-1}$

Propionic acid = 28.54 mmol l^{-1} → 2.85 mmol $bottle^{-1}$

Butyric acid = 6.12 mmol l^{-1} → 0.61 mmol $bottle^{-1}$

Total measured gas = 271.7 ml

Expected gas:

CO_2 = [CO_2 from Ac + CO_2 from Bu + CO_2 from Pr] x conversion factor to convert mmoles of gas to mls*

= (1.62 x 3.03) + (2.62 x 0.61) + (0.62 x 2.85) x 25.6

= 211.8 ml

(equation 5.1)

H_2 = [H_2 from Ac + H_2 from Bu + H_2 from Pr] x conversion factor

= (2 x 3.03) + (2 x 0.61) + (-1 x 2.85) x 25.6

= 113.4 ml

(equation 5.2)

Total gas = CO_2 + H_2 = 325.2 ml

*As the volume occupied by one mole of gas at 39 °C is equal to 25.6 litres (section 2.2.3.4) the value of the conversion factor required to convert mmoles of gas to ml of gas is also 25.6.

The predicted value was 120 % of the actual gas volume obtained experimentally (271.7 ml), hence the conversion of CO_2 and H_2 to CH_4 was considered:

$4\ H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ (equation 5.3)

As most of the H_2 produced from fermentation is expected to be involved in methanogenesis (Hungate, 1966), all of the H_2 was used in this calculation; that is, 113.4 ml. As 4 moles of H_2 combine with 1 mole of CO_2 to produce CH_4, the volume of CO_2 required to convert all 113.4 ml of H_2 to CH_4 was calculated by dividing 113.4 by 4 to give 28.35 ml CO_2;

113.4 ml H_2 + 28.35 ml $CO_2 \rightarrow$ 28.35 ml $CH_4 + H_2O$

Therefore the total gas = $CH_4 + CO_2 + H_2 = 28.35 + (211.8 - 28.35) + 0$

$= 211.8$ ml

This value is the same as the value calculated for CO_2 production alone. Hence calculation of CO_2 only may be sufficient for determining the volume of gas produced if all the H_2 is converted to CH_4. Most of the H_2 produced during rumen fermentation is expected to be involved in methanogenesis (Hungate, 1966; Russell & Wallace, 1997). Correspondingly measurements of H_2 concentration in the gas phase of rumen-like batch cultures have been low, for example, Von Grabe (1978) reported less than 1 % H_2 in the gas phase. More recently, Davies *et al.* (1997) found that even in the presence of bromoethanesulphonic acid (a chemical which inhibits the growth of methanogenic bacteria) less than 7 % of the total gas volume after incubation of wheat straw with ovine rumen fluid was accounted for by H_2, suggesting that where methanogenesis does not occur H_2 is used in the formation of other end - products.

Using the above procedure, the production of CO_2 calculated from theoretical considerations only accounts for 78 % of the actual experimental gas, therefore other

factors must affect the gas production. Production of minor VFA, such as iso-valeric and n-valeric acid, and 2- and 3-methylbutyric acids, may also contribute to the gas pool. Of the minor VFA, only iso- and n-valeric acids were measured in this experiment. No information on the stoichiometry of fermentation to iso- or n-valeric acid could be found, hence iso- and n-valeric acids were considered together as valeric acid, and it was assumed that the production of valeric acid was similar to the formation of propionic acid. That is, it did not result in the production of any fermentation gas, but did produce acidification gas (0.62 moles of gas for every mole of acid added). Calculating the volume of gas produced from valeric acid increased the volume of gas by 2.44 ml;

valeric acid at 60 h incubation of 1.0 g naked oats = 1.54 mmol l^{-1} $\rightarrow$ 0.154 mmol $bottle^{-1}$

Expected gas;

CO_2 = (CO_2 from valeric acid) x 25.6 = (0.62 x 0.154) x 25.6

= 2.44 ml (equation 5.4)

Hence, the new equation for predicting gas production becomes;

CO_2 = [(1.62 x Ac) + (2.62 x Bu) + (0.62 x Pr) + (0.62 x Val)] x 25.6

(equation 5.5)

This equation accounts for 79 % of the actual gas production measured during the incubation (improving the prediction by 1 %), leaving 21 % of the gas unaccounted.

Repeating the calculations for 1.0 g oatfeed and soya hulls after 60 h incubation resulted in predictions of 121.9 ml and 272.4 ml gas, respectively, accounting for 74 % and 82 % of the actual gas production for oatfeed and soya hulls respectively. As the gas production for oatfeed, naked oats and soya hulls cannot be predicted accurately from the production of acetic, propionic and butyric acids in terms of fermentation gas and acidification gas there must be other factors which affect gas production. Several possible explanations are discussed below;

1. The procedure used to estimate acidification gas may not have been appropriate: Mauricio *et al.* (1998) have demonstrated that both the quantity of VFA produced and the rate at which they are produced affects the rate and total volume of acidification gas produced. The problem imposed by acidification gas could be overcome by using a phosphate only buffer in the culture medium. This would result in the production of no acidification gas, however at present reports suggest that bicarbonate ions are necessary in order to sustain the rumen micro-organisms (Pell & Schofield, 1993).

2. Loss of volatile fatty acids from the liquid phase may have occurred by evaporation into the head-space: The quantity of VFA which would need to have diffused into the head-space to account for the difference in gas production between the predicted and the observed values was determined for 1.0 g naked oats at 60 h incubation (experiment 5.1.1) using equation 5.5 as follows;

Predicted gas = [(1.62 x 3.03) + (2.62 x 0.61) + (0.62 x 2.85) + (0.62 x 0.154)] x 25.6 = 214.2 ml
Actual gas production measured = 271.7 ml

Hence there is a difference of 57.5 ml between the predicted and the observed gas production.

Working the above equation backwards we can determine the amount of VFA required to produce 57.5 ml gas. If the VFA which diffuses into the head-space is produced in the same ratio as the VFA measured in the liquid phase then;

predicted gas = 214.2 ml / 25.6 = 8.37 moles
missing gas = 57.5 ml / 25.6 = 2.25 moles

2.25 / 8.37 = 0.27 (equation 5.6)

This value can then be used to determine the quantity of each VFA present and their respective gas production;

Ac → quantity present x 0.27 = 3.03 x 0.27 = 0.82

Bu → quantity present x 0.27 = 0.61 x 0.27 = 0.16

Pr → quantity present x 0.27 = 2.85 x 0.27 = 0.77

Val → quantity present x 0.27 = 0.154 x 0.27 = 0.04

(equation 5.7)

Expected gas

= [(1.62 x 0.82) + (2.62 x 0.16) + (0.62 x 0.77) + (0.62 x 0.04)] x 25.6

= [1.33 + 0.42 + 0.48 + 0.02] x 25.6

= 2.25 x 25.6

= 57.6 ml

Therefore an additional 2.25 mmol VFA are required to obtain the same amount of gas which was produced experimentally. This equates to a total VFA concentration in the gas phase of;

2.25 mmol VFA per 271.7 ml gas $\Rightarrow$ 0.008 mmol VFA ml^{-1} gas

or 0.22 mmol VFA mmol gas^{-1}.

Less than 1 % of the gas phase would therefore need to contain VFA in to account for the lost gas. The VFA have strong dissociation constants, with pKa values of approximately 4.8 (Argenzio & Stevens, 1984) and may therefore prefer to stay in solution. However the incubation temperature of 39 °C may encourage some diffusion into the head-space. In order to test this theory, the gas in the head-space of culture bottles should be analysed for VFA content and concentration.

3. Errors associated with stoichiometry: The stoichiometry described by Wolin (1960) and Hungate (1966) is based upon the fermentation of hexose. However as shown by the analysis of NSP (Table 5.1.1) the substrates used in this study contained pentose sugars as well as hexose sugars. The stoichiometry for hexose

sugars is based upon the major biochemical pathway used by rumen bacteria for hexose degradation, the Embden-Meyerhof-Parnas (Russell & Hespell, 1981). Information regarding the fermentation of pentoses is less well established. However using [1-^{14}C] arabinose, Turner and Roberton (1979) showed that pentose was fermented by *Bacteroides ruminicola* using a pentose phosphate cycle plus glycolysis to give product ratios similar to those obtained during the fermentation of hexoses. Leng (1973) also reported similarities in the end products obtained from the fermentation of pentose and hexose sugars, suggesting the following equations to account for their fermentation;

$$\text{Hexose} \rightarrow 2 \text{ pyruvate} + 4\text{ H} + 2\text{ ATP}$$

$$\text{Pentose} \rightarrow 1.67 \text{ pyruvate} + 1.67\text{ H} + 1.67\text{ ATP}$$

thereafter

$$2 \text{ pyruvate} + 2H_2O \rightarrow 2Ac + 2CO_2 + 2H_2 + 2ATP$$

$$2 \text{ pyruvate} + 8H \rightarrow 2Pr + 2H_2O + 2ATP$$

$$2 \text{ pyruvate} + 4H \rightarrow Bu + 2ATP + 2H_2 + 2CO_2$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O + ATP \qquad \text{(equation 5.8)}$$

Although the difference between pentose and hexose fermentation is small, it will affect the resulting VFA production. Gascoyne *et al.*, (1988), for example, observed an increase in propionate and valerate production when pentoses were added to hay and fermented by mixed ruminal micro-organisms.

4. Experimental error during gas production measurements: There will be a certain level of experimental error, in terms of both the equipment used and the human error involved. The error introduced by the equipment will be minimal, whilst the human error is likely to be larger. The main component of human error during gas production studies is likely to be the measurement of gas production throughout the incubation, i.e. associated with reading the volume of gas in the syringe. If the volume of gas is read to an accuracy of ± 0.5 ml throughout the incubation and fifteen readings are taken, the error associated with the total cumulative gas

production will be ± 7.5 ml. However, although worthy of comment, experimental error is unlikely to be responsible for all the difference noted between the predicted and experimental gas production volume.

5. Other possible factors which may have affected the amount of gas produced, and thus require further investigation, include gas production from minor VFA, the effect of ambient pressure on gas production readings and the effect of microbial interactions which may alter the expected amount of gas recorded. For example, the rumen bacteria, *Selenomonas ruminantium* and *Streptococcus bovis* can switch between the formation of VFA plus CO_2 and the formation of lactate minus CO_2, depending on their growth rate. This mechanism enables rapidly growing micro-organisms to maximise the ATP produced per unit of time instead of per unit of substrate (Russell & Wallace, 1997). There may also be other gases, not produced as a consequence of VFA production, in the culture bottles. For example, hydrogen sulphide and ammonia are often present in the rumen (Hungate, 1966) whilst water vapour from the culture medium will also be present (section 2.2.7.3.5).

The comparison of gas production profiles from different feedstuffs may be misleading as they are unlikely to be fermented to the same VFA ratio and this will affect the volume of gas which is produced (Table 5.1.11). One method of comparing gas production profiles may therefore be to adjust the VFA ratio for all feeds to a standard ratio. Although the ratio of VFA which is chosen may not be important it is necessary to correct the profiles of all feeds to this ratio in order to make meaningful comparisons. Correcting the gas production profiles of oatfeed, naked oats and soya hulls according to the VFA molar ratio produced after incubation of oatfeed resulted in the following gas volumes;

Experimental data (experiment 5.1.1):-

i. Molar percentage of VFA produced after incubation of oatfeed with a rumen microbial inoculum; 74.1 % Ac, 17.5 % Pr, 6.7 % Bu and 1.6 % Val.

ii. Total VFA production from naked oats = 66.51 mmol l^{-1} or 6.65 mmol $bottle^{-1}$.

Dividing the total VFA production from naked oats into the molar ratio for oatfeed resulted in the following values; 4.93 mmol Ac, 1.17 mmol Pr, 0.44 mmol Bu and 0.11 mmol Val. Using equation 5.5 to predict the theoretical gas production from this VFA production gave a predicted gas volume of 254.3 ml. Repeating this procedure for soya hulls resulted in a value for predicted gas production of 276.6 ml.

Therefore comparing all three feeds using a common VFA molar ratio would place the feeds in the following order from least to most degradable; oatfeed (123.5 ml), naked oats (254.3 ml) and soya hulls (276.6 ml). This however, is not the same as the order obtained from the experimental values for DM loss, where naked oats were degraded to a significantly greater extent than the soya hulls ($p < 0.01$; 978 mg g^{-1} for naked oats compared to 822 mg g^{-1} for soya hulls). This difference may be explained by the fermentation patterns of the micro-organisms during incubation of naked oats and soya hulls. For example, when micro-organisms such as *Selenomonas ruminantium* and *Streptococcus bovis* are grown rapidly under non-limiting conditions, such as with starch rich diets (for example naked oats), they produce lactate in addition to VFA and gas (Russell & Wallace, 1997). Thus when starch rich substrates are incubated during gas production studies, lactic acid may be produced initially, with VFA formation occurring after the rapidly fermentable components have been utilised (Beuvink & Spoelstra, 1992). The production of lactic or propionic acid during the initial stages of fermentation can be misleading as gas production profiles tend to show an enhanced lag phase or reduced initial growth rate when in fact microbial activity is high.

During the first 3 - 4 h of incubation, VFA molar proportions tend to vary greatly due to adaptation of the rumen microbial population to their new environment in the culture bottle. Relative to the total amount of VFA produced during the fermentation the quantity produced during the initial stages is quite small. However, this initial VFA production will play an important role in predicting the lag time and initial rate constant for the fermentation according to the various curve fitting procedures used to model gas production (France *et al.*, 1993).

From the above account, it will be clear that it is important to consider VFA profiles, and to adjust gas production profiles to a standard VFA profile, when comparing feeds with different VFA molar proportions or where fermentation patterns change during the course of fermentation. However, this should not be necessary when making comparisons between similar feedstuffs (Cone *et al.*, 1994).

Both Moseley and Jones (1984) and Mtengeti *et al.* (1995) have reported similar *in vitro* digestibilities between perennial ryegrass and white clover determined using the Tilley and Terry (1963) technique. However, for the samples used in this study DM loss after 145 h incubation was found to be greater from grass (905.7 mg g^{-1}) compared to clover (839.0 mg g^{-1}), although the total VFA production was similar, suggesting that DM loss should also have been similar. The total VFA produced during incubation of perennial ryegrass, white clover and various G:C mixtures was similar for all substrates, however the quantity of propionic and valeric acids differed, and hence the gas production profiles also differed between the different mixtures. When the gas production profiles for the different mixtures were added together in order to give values for a 50:50 grass:clover mixture (and hence similar VFA profiles), the cumulative gas production profiles were similar. In addition, predicting the gas production profiles from the different grass : clover mixtures by multiplying the 100 % grass and 100 % clover gas production profiles by the percentage of each in the mixture correlated well with the actual gas production profile for the different mixtures (R^2 = 0.999; Figure 5.1.8). This suggests that gas production from different mixtures can be predicted by addition of gas production profiles from the individual substrates, in their respective concentrations, thus removing the necessity of incubating several different mixtures. These findings are supported by Newbold *et al.* (1996) who examined the additivity of gas production estimates from a range of feedstuffs incubated individually and in a 50:50 ratio with wheat. The correlation between actual gas production and the predicted gas production for these mixtures was; actual = 0.07 + (0.93 x predicted), R^2 = 0.97, s.e. = 0.018. They also compared the total gas produced from more complex mixtures (8 commercial dairy compounds) with that predicted from incubation of the individual

components. For these complex mixtures they found that the actual gas production was 0.13 + (0.77 x predicted), $R^2 = 0.84$, s.e. = 0.021. Gas production profiles from individual feedstuffs may therefore be used to predict the gas production profiles for mixtures of feedstuffs and hence provide estimates for practical use in diet formulation. However this may not be the case for all feeds under all conditions and care should be taken when applying this to different mixtures. For example, some feedstuffs may compliment others so that gas production is greater than would be expected from the individual gas production profiles. Whilst others may have a detrimental effect on each other resulting in the production of less gas than would be expected from incubation of the individual components.

5.2 Gas production from the soluble fractions of naked oats (*Avena nuda*) and perennial ryegrass (*Lolium perenne*) hay

5.2.1 Introduction

Most feeds are composed of both a soluble and an insoluble fraction. The soluble fraction is believed to be readily degradable, and in the case of the *in situ* bag technique all soluble material is considered to be completely and instantaneously degraded in the rumen (Stern *et al.*, 1997). However, this is not the case for all soluble material. Soluble components contained in feeds include minerals, organic acids and various water soluble carbohydrates. The main water soluble carbohydrates in grass are glucose, fructose, sucrose and fructans (McDonald, 1973). Due to the importance of protein in ruminant diets in terms of rumen degradable and undegradable proteins (McDonald *et al.*, 1995) several studies have investigated the digestion of soluble and insoluble proteins. For example, Mahadevan *et al.* (1980) found that soluble feed proteins from casein, soybean meal, fish meal and rapeseed meal had different rates of degradation when treated with rumen proteases. Casein had the quickest rate of degradation, whilst the soluble protein from fish meal was degraded at twice the rate of that of either soybean or rapeseed meal. Different rates of degradation have also been observed for insoluble proteins. Prichard and Van Soest (1977) measured a rapidly degradable and a slowly degradable fraction in the insoluble protein component of several forages. A study into the degradability of soluble and insoluble proteins led Mahadevan *et al.* (1980) to conclude that solubility or insolubility was not the factor determining resistance or susceptibility to degradation rather the structural characteristics of the protein, for example, they found the presence of crosslinking disulphide bonds increased the resistance of feed protein to degradation. Wallace and Kopecny (1983) also speculated that the structure of the protein played an important part in determining the rate of digestion in the rumen. For example, they found that the diazotised casein protein, which exists in solution as a random coil, was readily digested, whilst the diazotised β - lactoglobulin protein, which has a more folded confirmation, was broken down more

slowly. The rate of digestion of gamma globulin and heme proteins was slowest of all and this was attributed to their highly ordered structures.

Gravimetric techniques for determining *in vivo* digestibility along with the *in situ* bag technique, are unable to measure the contribution of the soluble fraction to fermentation. The gas production technique, however, is able to measure the contribution of the soluble fraction to the fermentation (Pell & Schofield, 1993; Schofield & Pell, 1995b). The aim of this study was therefore to investigate the gas production from the soluble fractions of both naked oats and hay, in order to determine their rate of degradation and their contribution to the total gas pool.

5.2.2 Materials and Methods

Perennial ryegrass (*Lolium perenne*) hay (1.00 g x 6) and naked oats (*Avena nuda*) (1.00 g x 6) were used as the substrate (Appendix 1). Bottles numbered 1 - 6 contained 1.00 g hay, bottles 7 - 12 contained 1.00 g naked oats whilst bottles 13 - 15 were controls (contained no substrate). Bottles 4 - 6, 10 - 12 and 13 - 15 were prepared as for a normal gas production experiment (section 3.7) whilst bottles 1 - 3 and 7 - 9 were also prepared as normal with the exception that no reducing agent was added at this stage. All bottles were refrigerated at 4 °C for 9 h, then warmed to 39 °C. The contents of bottles 1 - 3 and 7 - 9 were then filtered through sinter glass crucibles of porosity 1 (as described for DM loss in section 3.10.1). The filtrate from each bottle was transferred into a clean bottle and gassed with CO_2 for 3 mins. Reducing agent (4 ml; section 3.7) was then added before the bottles were sealed and returned to the incubator (39 °C). Rumen microbial inoculum was then prepared from ovine rumen fluid (section 3.2.1 and 3.4) and each bottle inoculated with 10 ml of inoculum. Gas production was recorded at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 24, 30, 36, 42, 48, 54, 60 and 72 h. At the end of the incubation, pH, DM loss and VFA production were determined (section 3.10, 3.10.1 and 3.12.1, respectively).

The experiment was a factorial design consisting of 4 different substrates (hay, naked oats and the soluble fractions of both hay and naked oats), and 3 replicate bottles (4 x

3). The gas production profiles were fitted to the model of France *et al.* (1993) and compared using the parallel curve analysis function of MLP (Ross, 1987) (section 3.11). Values for the modelled gas production parameters, DM loss, VFA production and pH were analysed using analysis of variance in Genstat 5 (Lawes Agricultural Trust, 1993). Differences between treatments were calculated using the LSD (section 3.11).

5.2.3 Results

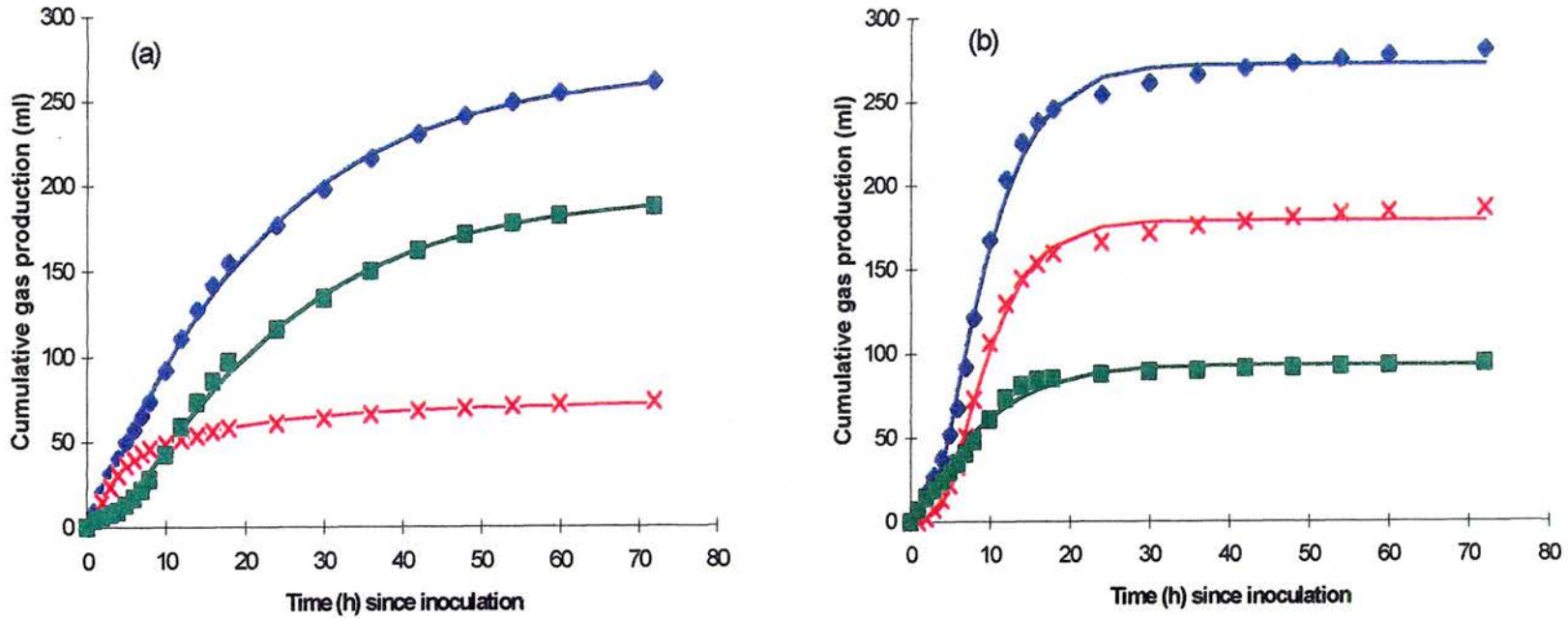
5.2.3.1 Quantification of the amount of soluble material present

The amount of soluble material present during the incubation (in bottles 1 - 3 and 7 - 9) was calculated as the total weight of substrate minus the residue remaining in the crucible after filtration. There was significantly less residue after filtration of the naked oats compared to that left for hay. Hence, 1.00 g naked oats contained more soluble material (695.1 ± 17.43 mg g^{-1}) than 1.00 g hay (279.7 ± 3.52 mg g^{-1}). There was therefore more substrate in the bottles containing the soluble component of 1.00 g naked oats than those containing the soluble component of 1.00 g hay at the start of the incubation.

5.2.3.2 Gas production

The gas production profiles for hay and naked oats together with the soluble fraction of naked oats and hay are shown in Figure 5.2.1. In order to estimate gas production from the insoluble fraction the gas production profile for the soluble fraction was subtracted from the gas production profile for the complete sample and these profiles are also shown in Figure 5.2.1. Gas production from the complete sample (soluble + insoluble fraction) resulted in the production of greater volumes of gas than from the soluble fraction alone, for both naked oats and hay. Parallel curve analysis of the gas production profiles for hay indicated significant differences, in both the rate and total volume of gas produced, between the gas production profiles for the soluble fraction and the complete hay sample ($p < 0.001$; Appendix 5.2.1.1). Parallel curve analysis also indicated a significant difference in the total volume of gas produced between the soluble fraction of naked oats and the complete naked oat sample, with more gas

Figure 5.2.1 Cumulative gas production profiles from the soluble fraction (-×-) and a whole sample (insoluble + soluble fraction) (-◆-) of (a) hay and (b) naked oats during incubation with a rumen microbial inoculum. The contribution of the insoluble component, determined as the difference between the whole and soluble fractions is also shown for both hay and naked oats (-■-).



Fermentations were conducted in 160 ml serum bottles with 89 ml of culture medium and 10 ml of rumen microbial inoculum. Each value represents the mean of three bottles, whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure. The contribution of the insoluble fraction was determined as the difference between the gas production profiles of the whole and soluble fractions.

being produced during incubation of the complete naked oats ($p < 0.001$). However, there was no significant difference in the rate of gas production between the soluble fraction and the complete naked oats (Appendix 5.2.1.2). There were also significant differences in both the total cumulative gas production and the rate of gas production between hay and naked oats ($p < 0.001$; Appendix 5.2.1.3) and also between the soluble fraction of hay and the soluble fraction of naked oats ($p < 0.001$; Appendix 5.2.1.3).

Fitted parameters and derived quantities for the gas production profiles are shown in Table 5.2.1. The rate of gas production, b and c, ranged from 0.0513 h^{-1} for the hay sample to 0.4980 h^{-1} for the soluble fraction of hay and from -1.262 $h^{-0.5}$ for the soluble fraction of naked oats to -0.0181 $h^{-0.5}$ for the soluble fraction of hay, for b and c respectively. The mean values of b and c across all samples were 0.3011 ± 0.09436 h^{-1} and -0.5228 ± 0.30402 $h^{-0.5}$, respectively. The total volume of gas produced was least during incubation of the soluble fraction of hay (79 ml), whilst most gas was produced during incubation of naked oats (273 ml). The mean total gas production was 200.1 ± 45.75 ml. The lag time (L_T) ranged from 0.10 h during incubation of hay to 2.83 h during incubation of the soluble fraction of naked oats. The time taken to produce 50 or 95 % of the total gas production, t_{50} and t_{95}, were both shorter for the soluble fraction of hay compared to the complete hay sample, whilst similar values were obtained between the soluble fraction of naked oats and the naked oat sample. The mean values for t_{50} and t_{95} across all samples were 9.85 ± 2.022 h and 36.94 ± 10.113 h, respectively.

Table 5.2.1 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for hay, naked oats (NO) and the soluble fractions of both hay (SFH) and naked oats (SFNO).

Gas production	Substrate				sed	Significance
parameters	SFH	Hay	SFNO	NO		
b (h^{-1})	0.4980^a	0.0513^b	0.3738^c	0.2815^d	0.0171	**
c ($h^{-0.5}$)	-0.0181^a	-0.0313^a	-1.2620^b	-0.7805^c	0.0907	**
A (ml)	79.0^a	269.0^b	179.6^c	272.8^b	4.00	***
B	120.8^a	264.5^b	61.7^c	154.8^d	5.34	***
L_T (h)	0.78^a	0.10^b	2.83^c	1.92^d	0.1529	**
t50 (h)	6.11^a	15.59^b	9.15^c	8.56^c	0.407	***
t95 (h)	43.01^a	62.93^b	20.34^c	21.47^c	0.583	***

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 5.2.1. Values in rows not bearing the same superscripts differ significantly (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The analysis of variance tables for the above data are shown in Appendix 5.2.2.1 - 5.2.2.7.

5.2.3.3 Dry matter loss

At the end of the incubation no particulate material was found in bottles containing the soluble fractions of hay (bottles 1- 3) and naked oats (bottles 7 - 9).

The average DM loss of the hay (bottles 4 - 6) was significantly lower (773.6 ± 7.48 mg g^{-1}), than for naked oats (bottles 10 - 12), where the average DM loss was 928.0 ± 6.06 mg g^{-1} ($p < 0.01$; Appendix 5.2.3).

5.2.3.4 Volatile fatty acid (VFA) production

The VFA production from incubation of hay, naked oats and the soluble fractions of both hay and naked oats are shown in Table 5.2.2. As expected, the total VFA production was significantly greater from the complete hay and naked oat samples (66.0 and 72.9 mmol l^{-1}, respectively) compared to both the soluble fraction of the

hay and the soluble fraction of the naked oats. Whilst, total VFA production from the soluble fraction of naked oats was significantly greater than that from the soluble fraction of hay (52.6 compared to 20.8 mmol l^{-1}; $p < 0.05$).

There were also differences in the molar percentages of VFA produced from the soluble fractions and the complete samples of hay and naked oats (Table 5.2.2). The highest percentage of Ac was seen during incubation of hay, followed by the soluble fraction of hay, with the lowest percentage of Ac produced from naked oats and the soluble fraction of naked oats ($p < 0.01$). The highest percentage of Pr was produced during incubation of the naked oats, whilst incubation of hay produced least ($p < 0.01$). The percentage of Bu was similar between hay, naked oats and the soluble fraction of hay, whilst incubation of the soluble fraction of naked oats resulted in a significantly higher percentage of Bu ($p < 0.01$). The percentage of Val was significantly lower after incubation of hay, compared to soluble hay, soluble naked oats and naked oats, which all produced similar proportions of Val ($p < 0.05$).

Table 5.2.2 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced from ryegrass hay (*Lolium perenne*), naked oats (NO; *Avena nuda*) and the soluble fractions of both hay (SFH) and naked oats (SFNO) after incubation with a rumen microbial inoculum.

	Substrate					
	SFH	Hay	SFNO	NO	sed	sig.
Total VFA (mmol l^{-1})	20.80^{a}	66.00^{b}	52.60^{c}	72.90^{b}	3.720	*
Acetate (molar %)	50.99^{a}	57.88^{b}	43.74^{c}	42.25^{c}	1.113	**
Propionate (molar %)	35.25^{a}	30.02^{b}	40.46^{c}	44.84^{d}	0.582	**
Butyrate (molar %)	9.82^{a}	9.52^{a}	12.29^{b}	9.55^{a}	0.355	**
Valerate (molar %)	3.93^{a}	2.58^{b}	3.50^{a}	3.43^{a}	0.314	*

Values in rows not bearing the same superscripts differ significantly (*$p < 0.05$; **$p < 0.01$). The analysis of variance tables are shown in Appendix 5.2.4.1 - 5.2.4.5.

5.2.3.5 Changes in batch culture pH

At the end of the incubation the pH was significantly higher in the bottles containing the soluble fraction of hay (pH 6.8), compared to those bottles containing the soluble fraction of naked oats (pH 6.6), which in turn was significantly higher than bottles containing either hay (pH 6.4) or naked oats (pH 6.4) ($p < 0.01$; Appendix 5.2.5).

5.2.4 Discussion

As expected gas production was greater from the complete (soluble + insoluble) sample compared to that from the soluble fraction alone, for both naked oats and hay. The contribution of the insoluble fraction, as determined by the difference between the gas production profiles for the complete sample and the soluble fraction indicated that the total gas production from the soluble fraction of naked oats was greater than that estimated for the insoluble fraction (180 ml versus 93 ml, respectively). Whilst the insoluble fraction of hay produced more gas than the soluble fraction (190 ml versus 79 ml, respectively). The greater gas production for the soluble fraction of naked oats compared to the insoluble fraction was expected as approximately 70 % of the naked oats appeared to be soluble, whilst only 28 % of the hay sample was soluble.

During this study, different VFA profiles were obtained during incubation of the complete naked oat and hay samples compared to those produced during incubation of their respective soluble fractions. The percentage of acetate produced during incubation of the complete hay sample was greater than that from the soluble fraction ($p < 0.01$), whilst the percentage of propionate and valerate were significantly greater after incubation of the soluble fraction ($p < 0.01$ and $p < 0.05$, respectively). For naked oats the percentages of acetate and valerate were similar after incubation of the soluble fraction and the complete sample whilst the percentage of propionate was significantly greater for the complete naked oats ($p < 0.01$) and the percentage of butyrate was significantly greater for the soluble fraction ($p < 0.01$). Conversely, for samples of clover, alfalfa, timothy and guinea grass, Schofield and Pell (1995b) reported that the acetate : propionate ratio did not differ between different fractions

of the same forage, although differences in VFA profiles were seen between the different forages. However, the VFA profiles were obtained from incubation of the complete forage and its insoluble NDF fraction rather than the soluble fraction. Stefanon *et al.* (1996) have investigated the VFA production from unfractionated (complete) samples of alfalfa and brome grass, and their respective insoluble and soluble fractions. However their statistical analysis looked at differences in the VFA profiles from the forages at different stages of maturity rather than differences in the VFA profiles produced from incubation of the different fractions. Using their data for VFA production from the different forage fractions at each stage of maturity, the percentage of the individual VFA was calculated and analysed by analysis of variance using Genstat 5 (Appendix 5.2.6.1 - 5.2.6.4). For both alfalfa and brome grass, the percentage of acetate, propionate and butyrate produced were similar between the unfractionated samples and the insoluble fractions, whilst the VFA molar percentages produced during incubation of the soluble fraction differed significantly ($p < 0.001$; Appendix 5.2.6.1 - 5.2.6.4).

The difference in gas production between the soluble fractions of naked oats and hay will be due to both the different proportion of soluble material present, 0.69 g versus 0.28 g for naked oats and hay respectively, and the respective VFA profiles. Therefore, to compare the soluble fractions of naked oats and hay on a similar basis both the VFA profiles and the amount of substrate present were standardised. In order to do this the following procedure was adopted. Firstly, equation 5.5 (section 5.1.4) was used to predict the theoretical gas production from the soluble fractions of both naked oats and hay;

soluble fraction of naked oats:

VFA production (mmol bottle^{-1}) = 2.30 Ac, 2.13 Pr, 0.65 Bu and 0.18 Val therefore

CO_2 = [(1.62 x 2.30) + (0.62 x 2.13) + (2.62 x 0.65) + (0.62 x 0.18)] x 25.6

= 175.6 ml

actual = 180 ml, hence predicted is only 2 % away from the actual

soluble fraction of hay:

VFA production (mmol $bottle^{-1}$) = 1.06 Ac, 0.73 Pr, 0.20 Bu and 0.08 Val therefore

CO_2 = [(1.62 x 1.06) + (0.62 x 0.73) + (2.62 x 0.20) + (0.62 x 0.08)] x 25.6

= 70.2 ml

actual = 79 ml, hence predicted is 11 % away from the actual

Secondly, the VFA molar ratios were corrected to those obtained from incubation of the soluble fraction of hay; the VFA molar ratio from incubation of the soluble fraction of naked oats was 44 Ac: 40 Pr: 12 Bu: 4 Val, whilst for the soluble fraction of hay the ratio was 51 Ac: 35 Pr: 10 Bu: 4 Val. When the total VFA production from the soluble fraction of naked oats was rearranged into the same molar proportions as those for the soluble fraction of hay, i.e. 52.6 mmol l^{-1} divided into 51 Ac: 35 Pr: 10 Bu: 4 Val, the gas production increased slightly;

soluble fraction of naked oats (corrected to the VFA profile obtained after incubation of the soluble fraction of hay):

VFA production (mmol $bottle^{-1}$) = 2.68 Ac, 1.84 Pr, 0.53 Bu and 0.21 Val therefore

CO_2 = [(1.62 x 2.68) + (0.62 x 1.84) + (2.62 x 0.53) + (0.62 x 0.21)] x 25.6

= 179.2 ml

The increase in gas production may not be as great as expected from the higher acetic acid and lower propionic acid concentrations (of the hay VFA profile) due to the slightly higher proportion of butyric acid present after incubation of naked oats. (Formation of butyric acid has the highest gas production of all the VFA).

Finally, the predicted gas production was corrected to 1.0 g of soluble material (to compare the gas production from similar quantities of soluble material);

0.28 g of the soluble fraction of hay theoretically produces 70.2 ml gas

$\Rightarrow$ 1.00 g soluble fraction hay will produce 250.7 ml gas

0.69 g soluble fraction of naked oats (producing the same VFA profile as the soluble fraction of hay) theoretically produces 179.2 ml gas

$\Rightarrow$ 1.00 g soluble fraction naked oats will produce 259.7 ml gas

Therefore, the soluble fractions of naked oats and oatfeed produce similar total volumes of gas (a difference of only 9 ml or 4 %) when 1.00 g of substrate is fermented to the same molar ratio.

This study has shown that the soluble fraction makes a significant contribution to the gas production profile and hence the digestion of the complete sample. All feeds contain various amounts of soluble material in the form of minerals, protein, organic acids and various simple sugars (Stefanon *et al.*, 1996), the quantity and composition of which will influence the resulting digestibility of the feedstuff. The soluble components have generally been considered to be readily digested by the micro-organisms of the rumen (Russell & Hespell, 1981; Chesson & Forsberg, 1997), however this may not be the case for all soluble feed components. For example, Neilson & Richards (1978) reported that in cattle fed a diet of the tropical grass *Heteropogon contortus* a large proportion of the lignin which was ingested was converted to a soluble lignin-carbohydrate complex which when in solution was not significantly affected by any further digestion in the rumen. Therefore feed evaluation techniques, such as the *in situ* technique, where all soluble material is considered to be instantaneously degraded may overestimate the degradability of feedstuffs. The gas production technique, however, measures the contribution of the insoluble and soluble fractions allowing a better understanding of feed degradation. In addition it allows the soluble and insoluble fractions to be compared on a similar basis (Stefanon *et al.*, 1996).

5.3 The effect of the source of inoculum on the digestion characteristics of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp.

5.3.1 Introduction

The microbial inoculum used in digestibility techniques has been reported to be the largest source of variation associated with these procedures (Weiss, 1994; Stern *et al.*, 1997). This variation can be attributed to animal to animal variation as well as to differences in donor animal species and the diet of the donor animal. The aim of this experiment was to investigate the gas production profiles from four feeds (oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp) using three different sources of inoculum (bovine rumen [BR], equine caecum [EC] and equine faecal [EF] inocula), and hence compare microbial digestion using microbial populations obtained from the bovine rumen and equine caecum.

Although the digestive tracts of the ruminant and the equidae have evolved through different evolutionary paths, the objective of both is to process forages efficiently (Janis, 1976). Like the rumen, the equine caecum contains anaerobic bacteria (Kern *et al.*, 1973 & 1974; Bonhomme-Florentin, 1988; Mackie & Wilkins, 1988), fungi (Orpin, 1981; Gold *et al.*, 1988) and protozoa (Kern *et al.*, 1973; Goodson *et al.*, 1988). The bacteria of both the rumen and the caecum can be classified into glycolytic, amylolytic, lactate utilisers, proteolytic, hemicellulolytic and cellulolytic bacteria (Julliand, 1992; Stewart *et al.*, 1997). Within these groups there are species of bacteria which are common to both the bovine rumen and the equine caecum, for example, *Streptococcus bovis*, *Bacteroides ruminicola*, *Bacteroides amylophilus* and *Lactobacillus* sp. (Kern *et al.*, 1973; Stewart *et al.*, 1997). Whilst there are similarities between the species of bacteria found in the rumen and the caecum, there do not appear to be any protozoan genera which are common to both species (Kern *et al.*, 1973 & 1974; Goodson *et al.*, 1988). There may be other micro-organisms which are common to both the bovine rumen and the equine caecum, however, knowledge of the micro-organisms present in the equine caecum is far from complete and behind that of the micro-organisms of the rumen (Julliand, 1992).

It is generally accepted that *in vivo* apparent digestibility is higher in ruminants than in equids (Haenlein *et al.*, 1966; Vander Noot & Gilbreath, 1970; Uden & Van Soest, 1982; Cymbaluk, 1990). Hyslop *et al.* (1997) suggested three hypotheses to explain these observations. Firstly, the rate of passage is faster in equids than in ruminants (Warner, 1981), hence the feed is subjected to the digestive actions of enzymes and micro-organisms for a shorter period of time. Secondly, there may be less opportunity for the absorption of microbial digestion end-products following hind gut fermentation in equids compared to foregut fermentation in ruminants, and finally, rumen micro-organisms may be more efficient at degrading fibrous feedstuffs than the caecal and colonic micro-organisms of equids.

The majority of micro-organisms present in the mammalian gastrointestinal tract are not attached to the gastrointestinal tract itself, but adhere either to food particles passing through the tract, or remain floating in the digesta. Hence, gut micro-organisms which are closely associated with plant debris in the rumen, are also excreted with plant residues in the faeces (Van Soest, 1982; Davies *et al.*, 1993a and b; Theodorou *et al.*, 1993; Nielsen *et al.*, 1995). Faecal material remains largely anaerobic after voiding, and the microflora can be viable for several hours after excretion from the digestive tract (Holter, 1991). Thus, faeces may provide an alternative source of inoculum for digestibility studies. Numerous authors have shown that the micro-organisms contained in the faeces, from both monogastric and ruminant animals, retain their ability to ferment fibre. Furthermore, faeces have been successfully used as a microbial inoculum for *in vitro* studies of feed degradability; these are summarised in Table 5.3.1.

Faeces from sheep and cattle have successfully replaced rumen inocula for *in vitro* studies of feed degradability (El Shaer *et al.*, 1987; Akhter *et al.*, 1994; Harris *et al.*, 1995). Whilst the use of human faeces as a microbial inoculum to investigate the digestibility of fibre, has also been widely documented (Ehle *et al.*, 1982b; McBurney & Thompson, 1987 & 1989; Mortensen *et al.*, 1988; Wedekind *et al.*, 1988; Adiotomre *et al.*, 1990; Vince *et al.*, 1990; Titgemeyer *et al.*, 1991). However,

reports on the use of equine faeces, as a source of microbial inoculum, for investigating the digestibility of equine feeds are scarce.

Table 5.3.1 A summary of *in vitro* studies that have used faecal material as the microbial inoculum to assess feed degradability.

	Animal	Authors
Monogastrics	cat	Sunvold *et al.*, 1994b & 1995
	dog	Sunvold *et al.*, 1994a & 1995
Ruminants	sheep	El Shaer *et al.*, 1987
	cattle	Akhter *et al.*, 1994
		Harris *et al.*, 1995

5.3.2 Materials and Methods

Oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp (0.75 g x 4 replicates) were incubated with bovine rumen (BR), equine caecum (EC) or equine faecal (EF) microbial inoculum. The chemical composition of the feedstuffs is shown in Appendix 1. In an attempt to obtain equal numbers of micro-organisms, DM values were determined for each of the three sources of inocula prior to the start of the incubation. Samples of each inoculum, before processing, were placed in dry, pre-weighed dishes. They were then weighed and dried at 80 °C to constant weight. 100 g samples of rumen fluid, caecal fluid and pony faeces were processed as described in section 3.4. Six 1 ml aliquots of each sample were then placed in dried, pre-weighed dishes, re-weighed and dried to constant weight at 80 °C. After drying the samples were re-weighed and dry matter determined. The DM of samples before processing were 15, 14 and 25 g kg^{-1} for bovine rumen, equine caecal and equine faecal material respectively. As the faecal material had approximately twice as much DM as the other inocula sources, the faecal inoculum was mixed with culture medium in a 50:50 ratio, to produce a DM content of 13 g kg^{-1}. Three identical series of bottles, each series consisting of 4 replicate bottles containing 0.75 g of oatfeed,

naked oats, soya hulls and unmolassed sugar beet pulp plus 4 substrate negative controls, were used. Bottles in series 1 were inoculated with BR, series 2 were inoculated with EC and series 3 were inoculated with EF. Gas production was recorded in all bottles at 3, 6, 9, 12, 16, 20, 24, 28, 32, 36, 44, 51, 58 and 72 h after inoculation. At the end of the incubation pH, DM loss, NSP loss and VFA production were determined (sections 3.10, 3.10.1, 3.12.1 and 3.12.3, respectively). The initial NSP composition of oatfeed, naked oats and soya hulls are shown in Table 5.1.1. The initial NSP composition of unmolassed sugar beet pulp was as follows; 137.90 mg arabinose, 136.78 mg glucose, 31.32 mg galactose, 6.53 mg mannose, 5.79 mg rhamnose, 8.14 mg xylose and 134.25 mg uronic acids, giving a total NSP content of 460.72 mg g^{-1} DM.

The experiment was a factorial design consisting of 4 different substrates, 3 different sources of inocula and 4 replicate bottles (4 x 3 x 4). The gas production profiles were fitted to the model of France *et al.* (1993) and compared using the parallel curve analysis function of MLP (Ross, 1987) (section 3.11). Values for the modelled gas production parameters, DM loss, VFA production and pH at the end of the incubation were analysed using analysis of variance in Genstat 5 (Lawes Agricultural Trust, 1993). Differences between treatments were calculated using the LSD (section 3.11).

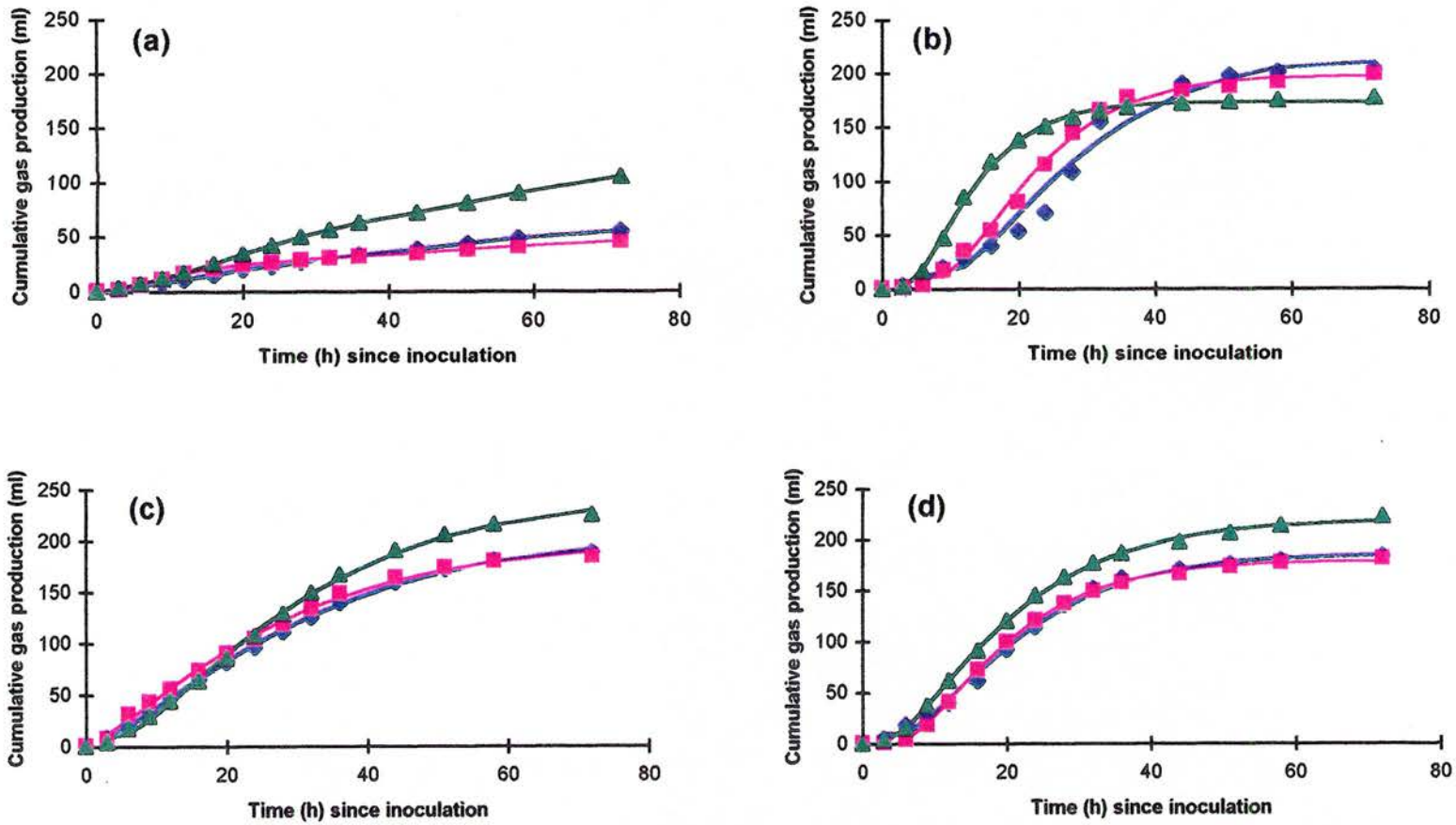
5.3.3 Results

5.3.3.1 Gas production

Figure 5.3.1 shows the gas production profiles obtained during the incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp with either equine caecal (EC), equine faecal (EF) or bovine rumen (BR) fluid inoculum.

Parallel curve analysis indicated significant differences in gas production during incubation of oatfeed with EC, EF and BR ($p < 0.001$; Appendix 5.3.1). Most gas was produced when oatfeed was incubated with BR (105 ml) followed by EC (55 ml) with least gas being produced during incubation with EF (44 ml). Incubation of naked oats with EC and EF produced similar quantities of gas (221 and 200 ml for

Figure 5.3.1 Cumulative gas production profiles for **(a)** oatfeed, **(b)** naked oats, **(c)** soya hulls and **(d)** unmolassed sugar beet pulp with either an equine caecal (-◆-), equine faecal (-■-) or a bovine rumen (-▲-) microbial inoculum.



Fermentations were conducted in 160 ml serum bottles with 89 ml of culture medium and 10 ml of either equine caecal, equine faecal or bovine rumen microbial inoculum. Each value represents the mean of four bottles, whilst (with the exception of oatfeed) the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure.

EC and EF, respectively) and less gas was produced during incubation with BR (173 ml). The rates of gas production were also significantly different between the inocula ($p < 0.05$). Incubation of soya hulls with EC and BR resulted in similar rates of gas production, although significantly more gas was produced during incubation with BR ($p < 0.001$). Incubation of soya hulls with EF resulted in the production of least gas ($p < 0.01$). There were no significant differences between the gas production profiles obtained during incubation of unmolassed sugar beet pulp with either EC or EF, whilst incubation of unmolassed sugar beet pulp with BR produced significantly more gas ($p < 0.001$) at a significantly quicker rate of gas production ($p < 0.05$).

Fitted parameters and derived quantities for the gas production profiles are shown in Table 5.3.2. Oatfeed did not reach an asymptote of gas production within the 72 h incubation period with any of the microbial inoculum, and the model of France *et al.* (1993) could not be used to produce reliable data. For the remaining feeds (naked oats, soya hulls and unmolassed sugar beet pulp) the rates of gas production, b and c, ranged from 0.0492 h^{-1} (for soya hulls incubated with EC) to 0.2241 h^{-1} (for naked oats incubated with BR) and from -0.9570 $h^{-0.5}$ (for naked oats incubated with EF) to -0.1130 $h^{-0.5}$ (for soya hulls incubated with EF), respectively. The mean values for b and c across all feeds and inocula were 0.1161 ± 0.01974 h^{-1} and -0.4802 ± 0.09788 $h^{-0.5}$. The predicted asymptote of gas production, A, for all feeds and all inocula ranged from 173 ml (for naked oats incubated with BR) to 245 ml (for soya hulls incubated with BR), with a mean value of 203 ± 7.62 ml. Both the shortest and longest lag times, L_T, were seen during incubation with EF. The lag time ranged from 1.23 h (for soya hulls incubated with EF) to 7.20 h (for naked oats incubated with EF), with a mean value across all feeds and inocula of 3.93 ± 0.643 h. The time taken to produce 50 % of the total gas production, t_{50}, ranged from 12.56 h (for naked oats incubated with BR) to 26.53 h (for naked oats incubated with EC), with a mean value across all feeds and inocula of 21.22 ± 4.536 h. The time taken to produce 95 % of the total gas production, t_{95}, ranged from 29.70 h (for naked oats incubated with BR) to 84.19 h (for soya hulls incubated with EC), with a mean value across all feeds and inocula of 57.76 ± 6.031 h.

Table 5.3.2 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for naked oats (NO), soya hulls (SH) and unmolassed sugar beet pulp (SB) incubated with either an equine caecal (EC), equine faecal (EF) or a bovine rumen (BR) fluid inoculum.

Gas production parameter	Substrate	Inoculum			
		EC	EF	BR	s.e.d
b (h^{-1})	NO	0.1140^{a}	0.1787^{b}	0.2241^{c}	
	SH	0.0492^{d}	0.0509^{d}	0.0630^{d}	
	SB	0.1202^{a}	0.1428^{e}	0.1017^{a}	0.0088
c ($h^{-0.5}$)	NO	-0.6220^{a}	-0.9570^{b}	-0.7970^{c}	
	SH	$-0.1400^{d,e}$	-0.1130^{d}	-0.2390^{e}	
	SB	-0.5070^{f}	$-0.6010^{a,f}$	-0.3460^{g}	0.0504
A (ml)	NO	218.98^{a}	197.49^{b}	173.10^{c}	
	SH	208.96^{d}	200.48^{b}	244.93^{e}	
	SB	185.99^{f}	$178.19^{c,f}$	220.33^{a}	3.968
B	NO	111.59^{a}	53.20^{b}	84.80^{c}	
	SH	179.83^{d}	$181.24^{d,e}$	188.71^{e}	
	SB	104.40^{a}	95.18^{f}	162.08^{g}	3.787
L_T (h)	NO	6.36^{a}	7.20^{b}	3.18^{c}	
	SH	2.03^{d}	1.23^{e}	3.59^{f}	
	SB	4.45^{g}	4.43^{g}	2.89^{c}	0.1804
t_{50} (h)	NO	26.53^{a}	21.30^{b}	12.56^{c}	
	SH	25.47^{a}	22.07^{b}	26.29^{a}	
	SB	19.72^{d}	18.62^{d}	18.41^{d}	0.756
t_{95} (h)	NO	61.45^{a}	45.92^{b}	29.70^{c}	
	SH	84.19^{d}	76.38^{e}	76.60^{e}	
	SB	50.08^{f}	44.73^{b}	50.75^{f}	2.041

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 5.3.1. Values for each parameter not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables are shown in Appendix 5.3.2.1 - 5.3.2.7.

5.3.3.2 Dry matter loss

DM loss after 72 h incubation varied for the different feeds and with the different microbial inocula (Table 5.3.3). However, the pattern of DM loss for corresponding feeds was similar between all inocula with naked oats being degraded to the greatest extent, followed by soya hulls, then sugar beet with oatfeed being the least degradable substrate. DM loss from oatfeed was similar after incubation with EC (305.3 ± 6.34 mg g^{-1}) and EF (291.9 ± 3.02 mg g^{-1}), whilst incubation with BR resulted in a significantly higher value for DM loss (450.3 ± 6.72 mg g^{-1}; $p < 0.05$). DM loss from naked oats was significantly higher after incubation with BR (972.6 ± 2.93 mg g^{-1}) compared to incubation with EC (956.2 ± 6.98 mg g^{-1}; $p < 0.05$). Whilst there was no difference in DM loss after incubation with EF (964.3 ± 2.23 mg g^{-1}) compared to either incubation with EC or BR. Most DM loss from soya hulls and unmolassed sugar beet pulp occurred during incubation with BR (956.7 ± 1.03 mg g^{-1} and 900.3 ± 6.63 mg g^{-1}, respectively), with significantly less DM being degraded during incubation with EC ($p < 0.05$; 886.3 ± 1.97 mg g^{-1} and 857.2 ± 14.75 mg g^{-1} for soya hulls and sugar beet respectively), and least from incubation with EF ($p < 0.05$; 806.6 ± 2.17 mg g^{-1} and 798.2 ± 28.85 mg g^{-1}, respectively).

Table 5.3.3 Dry matter loss (mg g^{-1}) of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp after 72 h incubation with a microbial inoculum prepared from either equine caecal (EC), equine faecal (EF) or bovine rumen fluid (BR).

Inoculum	Feed			
	Oatfeed	Naked oats	Soya hulls	Sugar beet
EC	305.3^{a}	956.2^{b}	886.3^{d}	857.2^{e}
EF	291.9^{a}	$964.3^{b,c}$	806.6^{f}	798.2^{f}
BR	450.3^{g}	972.6^{c}	956.7^{b}	900.3^{h}

Values not bearing the same superscripts differ significantly ($p < 0.05$). The s.e.d. was 5.30. The analysis of variance table is shown in Appendix 5.3.3.

5.3.3.3 Loss of non-starch polysaccharide (NSP)

Values for the loss of NSP from oatfeed, soya hulls and unmolassed sugar beet pulp after 60 h incubation with EC, EF and BR are shown in Table 5.3.4. Data for all naked oat samples and some samples of soya hulls and unmolassed sugar beet pulp were not determined as there was insufficient sample remaining at the time of harvest for NSP analysis. Thus the loss of NSP from naked oats during incubation with all inocula and from both soya hulls and unmolassed sugar beet pulp during incubation with EC were considered to be 1000 mg g^{-1}.

Loss of NSP from oatfeed was greatest during incubation with BR (494.44 mg g^{-1}), whilst incubation with EC and EF resulted in a similar loss of oatfeed NSP (310.01 and 279.63 mg g^{-1}, for EC and EF respectively). The uronic acid component of oatfeed was degraded to the greatest extent by all inocula (489.38, 457.94 and 628.72 mg g^{-1} uronic acid for EC, EF and BR, respectively) whilst glucose was degraded least (244.64, 210.44 and 472.23 mg g^{-1} glucose for EC, EF and BR, respectively). Loss of NSP from soya hulls was considered to be 1000 mg g^{-1} during incubation with EC, whilst incubation with EF and BR also resulted in high losses of NSP (913.79 and 971.89 mg g^{-1}, for EF and BR, respectively). During incubation of soya hulls with EF, glucose was degraded to the greatest extent (959.28 mg g^{-1} glucose), whilst rhamnose was degraded to the least extent (-0.40 mg g^{-1}; this negative degradability suggests there may have been microbial contamination of the residue). The arabinose, galactose, mannose and rhamnose components of soya hulls all appeared to be degraded completely (1000 mg g^{-1}) after incubation with BR. Degradation of the remaining NSP constituents were also high with xylose being degraded to the least extent (948.03 mg g^{-1} xylose). The NSP components of unmolassed sugar beet pulp were considered to be completely degraded during incubation with EC, as there was not enough residue for analysis. Whilst incubation with EF and BR resulted in the loss of 913.79 and 971.89 mg g^{-1} NSP, respectively. During incubation with EF, mannose and rhamnose were degraded to the greatest extent (1000 mg g^{-1}), whilst the xylose component was least degradable (718.67 mg g^{-1} xylose). The arabinose, galactose, mannose, rhamnose and xylose components of

Table 5.3.4 Loss of non-starch polysaccharide components from oatfeed, soya hulls and unmolassed sugar beet pulp (mg g^{-1} NSP or NSP constituents) following a 60 h incubation with either an equine caecal (CFI), equine faecal (PFI) or bovine rumen (RFI) fluid microbial inoculum.

Substrate		Loss of NSP components (mg g^{-1} NSP component)							Loss of total
Inocula		Arabinose	Glucose	Galactose	Mannose	Rhamnose	Xylose	UAC	NSP (mg g^{-1} NSP)
Oatfeed	CFI	353.91	244.64	343.67	-	-	359.63	489.38	310.01
	PFI	320.80	210.44	309.97	-	-	333.76	457.94	279.63
	RFI	492.17	472.23	491.91	-	-	509.19	628.72	494.44
Soya hulls	CFI								
	PFI	846.15	959.28	720.79	875.04	-0.40	850.47	949.07	913.79
	RFI	1000.00	963.96	1000.00	1000.00	1000.00	948.03	976.05	971.89
Sugar beet	CFI								
	PFI	983.83	945.24	961.05	1000.00	1000.00	718.67	991.13	968.70
	RFI	1000.00	984.79	1000.00	1000.00	1000.00	1000.00	984.88	991.08

No values are shown for naked oats, nor for soya hulls and unmolassed sugar beet pulp incubated with CFI, due to insufficient residue remaining after incubation for NSP analysis. Where a degradation of 1000 mg g^{-1} is shown the component could not be detected in the residue.

the unmolassed sugar beet pulp were degraded to the greatest extent by the BR (1000 mg g^{-1}), whilst degradation of the glucose and uronic acid components was also high (984.79 and 984.88 mg g^{-1} for glucose and uronic acid respectively).

5.3.3.4 Volatile fatty acid (VFA) production

VFA production varied between feeds and with the various sources of inoculum (Table 5.3.5). During incubation with both EC and BR total VFA production was greatest for soya hulls, followed by unmolassed sugar beet pulp and naked oats, with least produced after incubation of oatfeed. Incubation with EF resulted in the following order of VFA production, from most to least; unmolassed sugar beet pulp > soya hulls and naked oats > oatfeed. For all feeds, incubation with BR generally resulted in the greatest quantities of total VFA. Incubation of oatfeed and naked oats with EC and EF resulted in similar levels of total VFA, whilst for soya hulls total VFA production was greater with EC and for unmolassed sugar beet pulp incubation with EF produced more total VFA than incubation with EC. Total VFA production across all feeds for all inocula sources ranged from 11.89 mmol l^{-1} during incubation of oatfeed with EF to 51.74 mmol l^{-1} during incubation of soya hulls with BR. The mean total VFA production for all feed and inocula sources was 36.89 ± 3.939 mmol l^{-1}.

There were also significant differences in the VFA molar percentages produced during incubation of the various feeds with the different inocula (Table 5.3.5). The percentage of acetate produced by the feedstuffs during incubation with EC and EF was greatest for unmolassed sugar beet pulp and least after incubation of naked oats. During incubation with BR, the greatest percentage of acetate was produced from oatfeed and the least from naked oats. There were significant differences in the percentage of acetate produced during incubation with the different inocula for all feeds, except unmolassed sugar beet pulp where the percentage of acetate produced was similar after incubation with EC, EF and BR. Acetate production across all feedstuffs and inocula sources ranged from 29.39 % during incubation of naked oats with EC to 67.45 % during incubation of oatfeed with BR, with a mean value of

50.15 ± 3.458 %. For all inocula the percentage of propionate produced was greatest during incubation of naked oats. The lowest percentage of propionate, after incubation with EC and EF, was from unmolassed sugar beet pulp, whilst incubation of oatfeed produced the lowest percentage of propionate of all the feeds incubated with BR. For both oatfeed and soya hulls the percentage of propionate was greatest after incubation with EC and EF compared to incubation with BR, whilst the opposite was seen for naked oats and unmolassed sugar beet pulp; incubation with BR producing higher percentages of propionate than incubation with either EC or EF. The percentage of propionate produced from all feeds with all sources of inoculum ranged from 23.97 % during incubation of oatfeed with BR to 58.14 % during incubation of naked oats with BR. The mean propionate production for all feeds and sources of inoculum was 36.78 ± 2.622 %. During incubation of all feedstuffs with all inocula the percentage of butyrate produced was generally greatest for naked oats and lowest after incubation of oatfeed, whilst incubation with EC and EF generally resulted in higher percentages of butyrate than incubation with BR. For all feeds and inocula sources butyrate production ranged from 7.45 % during incubation of oatfeed with BR to 22.92 % during incubation of naked oats with EF, with a mean value of 11.58 ± 1.435 %. Incubation with EC and EF ranked the feedstuffs in the following order in terms of most to least valerate production; naked oats > soya hulls > unmolassed sugar beet pulp > oatfeed, whilst incubation with BR resulted in the order, soya hulls > unmolassed sugar beet pulp > oatfeed > naked oats. For oatfeed, soya hulls and unmolassed sugar beet pulp a higher percentage of valerate was produced during incubation with BR than either EC or EF, whilst for naked oats incubation with EC produced a higher percentage of valerate than incubation with EF, which in turn produced more than incubation with BR. The percentage of valerate produced from all feedstuffs incubated with all inocula ranged from 0 % during incubation of oatfeed with EF to 4.76 % during incubation of naked oats with EC, with a mean value of 1.57 ± 0.361 %.

Table 5.3.5 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced from oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp following 72 h incubation with equine caecal (EC), pony faecal (EF) or rumen fluid (BR) inoculum.

VFA	Inocula	Oatfeed	Naked oats	Soya hulls	Sugar beet	s.e.d.
Total VFA	EC	12.41^{a}	41.02^{c}	47.88^{e}	41.56^{c}	
(mmol l^{-1})	EF	11.89^{a}	39.72^{c}	41.81^{c}	44.64^{d}	
	BR	21.38^{b}	44.14^{d}	51.74^{f}	$44.55^{c,d}$	1.468
Acetate	EC	48.21^{a}	29.39^{d}	48.52^{g}	58.33^{j}	
(molar %)	EF	53.59^{b}	35.29^{e}	53.61^{h}	58.26^{j}	
	BR	67.45^{c}	32.28^{f}	60.43^{i}	56.46^{j}	0.947
Propionate	EC	$39.50^{a,e}$	45.08^{c}	41.31^{e}	30.32^{g}	
(molar %)	EF	38.96^{a}	39.18^{a}	33.60^{f}	30.17^{g}	
	BR	23.97^{b}	58.14^{d}	28.64^{g}	32.53^{h}	0.959
Butyrate	EC	12.04^{a}	20.77^{c}	$8.49^{b,f}$	$10.18^{e,f}$	
(molar %)	EF	$8.72^{b,f}$	22.92^{d}	10.89^{e}	$10.55^{e,g}$	
	BR	7.45^{b}	$8.69^{b,f,g}$	$8.76^{b,f,g}$	$9.55^{e,f}$	0.956
Valerate	EC	0.24^{a}	4.76^{c}	$1.67^{e,g}$	$1.17^{b,g}$	
(molar %)	EF	0.00^{a}	2.61^{d}	1.91^{e}	1.01^{b}	
	BR	1.07^{b}	0.88^{b}	2.14^{f}	1.43^{g}	0.149

Values for total VFA and individual acids not bearing the same superscripts differ significantly ($p < 0.05$). Analysis of variance tables are shown in Appendix 5.3.4.1 - 5.3.4.5.

5.3.3.5 Changes in batch culture pH

There were significant differences in the pH of the culture medium after incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp with either EC, EF or BR (Table 5.3.6).The pH after incubation of oatfeed ranged from 6.70 with BR to 6.81 with EF, whilst for bottles containing naked oats the pH ranged from 6.56 with BR to 6.68 with EF. Incubation of soya hulls resulted in a pH range of 6.58 for BR to 6.64 with EF, whilst for unmolassed sugar beet pulp the pH ranged from 6.56 with

EC to 6.61 with BR. The largest difference in pH was between bottles containing unmolassed sugar beet pulp with EC (6.56) and those containing oatfeed inoculated with EF (6.81). The largest difference in pH at the end of the incubation was approximately 0.2 pH units, which is unlikely to have a biologically significant effect on the incubation.

Table 5.3.6 pH after incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal (EC), equine faecal (EF) or bovine rumen (BR) fluid inoculum.

Inocula	Feed			
	Oatfeed	Naked oats	Soya hulls	Sugar beet
EC	6.78^{a}	6.65^{d}	6.61^{g}	6.56^{f}
EF	6.81^{b}	6.68^{e}	6.64^{h}	6.60^{g}
BR	6.70^{c}	6.57^{f}	6.58^{i}	6.61^{g}

Values not bearing the same superscripts differ significantly ($p < 0.05$). The s.e.d was 0.00757. The analysis of variance table is shown in appendix 5.3.5.

5.3.4 Discussion

Of the inocula used in this study, BR appeared to be the most effective at degrading the fibrous feedstuffs (oatfeed, soya hulls and unmolassed sugar beet pulp), whilst all three inocula degraded naked oats to a similar extent. Koller *et al.* (1978) reported similar findings when investigating the digestibility of timothy hay, orchard grass, wheat straw and alfalfa between ruminants and equines using the *in vitro* technique of Goering and Van Soest (1970). They found that the digestibility of timothy hay, orchard grass and wheat straw was greater when the feedstuffs were incubated with ruminal micro-organisms compared to equine micro-organisms, whilst there were no differences in the digestibility of alfalfa. Similar findings have also been reported *in vivo*, for example, Haenlein *et al.* (1966), Vander Noot and Gilbreath (1970) and Cymbaluk (1990) have all reported better digestion of fibrous feedstuffs by cattle compared with horses; whilst less fibrous feedstuffs may be digested to a similar extent. However Alexander (1963) and Dr. V. Julliand (INRA, France - personal

communication) found that when degradation in the bovine rumen and equine caecum were compared by suspending a cotton thread in the rumen of a steer and the caecum of a pony, the thread was degraded faster in the caecum than the rumen. This discrepancy in results may be due to the methods used and the form of cellulose being investigated; cotton thread is a pure, unlignified crystalline form of cellulose whereas cellulose in plant cell walls is found closely associated with other substances, such as, hemicelluloses. Therefore the degradation of cotton thread may not be representative of the ability of the rumen and the caecum to degrade fibrous feedstuffs. However, using the *in situ* bag technique Hyslop *et al.* (1997), also reported greater degradation of substrates in the equine caecum compared to the bovine rumen. They found unmolassed sugar beet pulp and hay cubes were degraded to a greater extent during the initial 12 h of incubation in the equine caecum compared with incubation in the bovine rumen. Whilst at 48 h incubation CP and ADF degradation were similar between the two species. They concluded that differences in apparent digestibility *in vivo* between equids and ruminants could not be explained by assuming that the caecal micro-organisms of equids are less efficient at degrading fibrous feedstuffs than rumen micro-organisms. The difference between the observations of Hyslop *et al.* (1997) and this study suggest that, although caecal micro-organisms are able to degrade the feedstuff when incubated under the conditions described for the pressure transducer technique, these conditions are perhaps not ideal for the caecal micro-organisms and hence they do not function at their maximum level of activity. There are two factors which may disadvantage the caecal micro-organisms in this technique; firstly the buffered salt solution used in this study was based upon simulation of the composition of ruminant saliva (as described by McDougall, 1949), although equine saliva is similar to ruminant saliva there are differences, for example, the sodium and phosphate, but not chloride, contents of equine saliva may be less than that in ruminant saliva (Eckersall *et al.*, 1985). Secondly, as equines are hindgut fermenters the amount of saliva which reaches the caecum will be much smaller than that which reaches the rumen and the culture medium may therefore not be entirely suitable for the caecal micro-organisms (Argenzio, 1993).

Cattle faeces, one to seven days after voiding, have been shown to contain 1 - 2 % oxygen, 20 - 30 % carbon dioxide and 30 - 50 % methane (Holter, 1991). Thus faecal material remains largely anaerobic after voiding, and the microflora can be viable for several hours after excretion from the digestive tract (Holter, 1991; Theodorou *et al.*, 1993). El Shaer *et al.* (1987) found that faeces voided one to six hours prior to use as a microbial inoculum gave values for *in vitro* digestibility which were indistinguishable from those obtained using faeces which had been collected within one hour of voiding. However, in this study faeces were collected within one hour of voiding to try and maximise microbial activity.

The number of micro-organisms present in a sample of ruminal, caecal or faecal material is difficult to quantify and it was therefore decided to standardise the microbial inocula on the basis of DM. This provided a rough estimate of microbial mass, although the DM of micro-organisms could not be distinguished from the DM of any feed particles present in the digesta and faeces. Rumen and caecal digesta had similar DM after processing (15 and 14 g kg^{-1} DM, respectively), whilst the faecal sample had a DM content approximately double that of the rumen and caecal digesta (25 g kg^{-1} DM). The faecal inocula was therefore diluted (1:1) with culture medium (section 3.7). The major problem with this approach is its non-specificity, however both diluted ovine and bovine faecal inoculum have been successfully used during fermentation studies (El Shaer *et al.*, 1987; Harris *et al.*, 1995).

Incubation of the feeds with EC generally produced more gas than when the feeds were incubated with EF. However, Harris *et al.* (1995) found the opposite occurred when they compared rumen digesta with faeces from a lactating dairy cow. Incubation of their test feeds (winter beans, spring barley, soya bean meal and rapeseed meal) with faecal material generally resulted in higher gas production levels than during incubation with BR. The rate of gas production was also higher with faecal inocula during the later stages of fermentation. They concluded that faecal micro-organisms were better adapted than rumen micro-organisms to ferment slowly digestible feed components.

The three inoculum sources and the three feedstuffs used in this study resulted in the production of different VFA profiles, highlighting the importance of standardising gas production curves with respect to the VFA profile. The different VFA profiles suggest that either different groups of micro-organisms were responsible for degrading the substrates or that different pathways were favoured for substrate degradation in the various inocula. The NSP from naked oats, sugar beet and soya hulls were all highly degraded by the various inocula. Incubation of oatfeed with EC and EF resulted in similar losses of the NSP components, although incubation with EC generally resulted in higher losses than incubation with EF. Whilst incubation with BR resulted in the highest NSP loss from oatfeed. These higher losses with BR agree with the *in vitro* and *in vivo* observations that ruminants are better able to degrade fibrous components than equines (Koller *et al.*, 1978; Haenlein *et al.*, 1966; Vander Noot & Gilbreath, 1970; Cymbaluk, 1990).

Faeces, as the microbial inoculum for *in vitro* digestibility studies with equines have several advantages over caecal digesta. Faeces are a readily available source of micro-organisms, do not require the use of surgically prepared (fistulated) animals, and can be collected from any individual or several animals (thereby minimising the effects of animal to animal variation). A faecal inoculum may also provide the opportunity of studying fermentation in horses with compromised gut function, such as in animals with chronic diarrhoea. The differences in gas production profiles that result from incubation of faeces compared with caecal or rumen inocula necessitate the use of separate prediction equations in order to predict *in vivo* digestibility from *in vitro* data.

CHAPTER 6 - THE EFFECT OF SAMPLE PROCESSING ON GAS PRODUCTION

6.1 Introduction

During both *in vitro* and *in situ* digestibility studies, feeds are usually dried (in an oven or freeze drier), ground through a 1 or 4 mm dry mesh screen, then incubated with a sample of rumen micro-organisms. Drying the feeds enables them to be stored and used as and when required. It also enables preparation of representative (homogenous) samples. With wet (fresh) samples, digestibility measurements have to be made within a short time of harvesting and sampling the forage. In addition, wet samples are difficult to grind and often result in a non-uniform preparation consisting of coarse particles which are difficult to work with and increase the variation in the estimation of rumen degradability (Lopez *et al.*, 1995). Although, fresh samples do promote problems, many feeds are generally ingested in their fresh state and estimating the degradability of a feed by incubating dried samples may be misleading. In addition, the drying technique used may reduce the organic matter (Acosta & Kothmann, 1978), total non-structural carbohydrates (Acosta & Kothmann, 1978) and protein solubility (Abdalla *et al.*, 1988) of the feed while increasing its apparent lignin content (Burritt *et al.*, 1988). Where plant enzyme activity is thought to contribute to the digestion process in the rumen (M.K. Theodorou - personal communication), drying of samples will also destroy this activity.

Although grinding reduces variation in the estimation of rumen degradability, this too may cause problems as rumen micro-organisms, in their natural environment, tend to colonise larger particles. During a feeding bout, cattle, for example, tend to ingest large mouthfuls of food which pass to the rumen with little chewing (Dulphy *et al.*, 1980; Phillips, 1993; Hobson, 1997). Sheep, although more selective in their feeding behaviour than cattle, also show less chewing movements during feeding bouts than non-ruminant animals, such as horses (Dulphy *et al.*, 1997; Lynch *et al.*, 1992). Therefore, the vegetation which enters the rumen will have undergone minimal

mechanical breakdown. For example, when Poppi *et al.* (1981b) fed chopped Pangola (*Digitaria decumbens*) and Rhodes (*Chloris gayana*) grass stems containing 0.86 g g^{-1} of large particles (> 1.18 mm), mastication by cattle reduced the proportion of large particles to 0.76 g g^{-1}, whilst mastication by sheep reduced the proportion of large particles to 0.67 g g^{-1}. Thus, after a feeding bout the rumen will contain particles that are considerably larger than the dried and ground particles commonly used in feed evaluation studies. The exact size of the particles which enter the rumen after a feeding bout is affected by the chemical composition and structure of the forage (Lee & Pearce, 1984; McLeod *et al.*, 1990), the initial particle size of the feed (Bailey *et al.*, 1990) and the quantity of feed eaten (Dulphy *et al.*, 1980). The particle size may be reduced further by re-chewing or rumination at certain time intervals after the feeding bout has finished. This involves muscular contractions of the rumen which cause individual feed boluses to move back up the digestive tract to the mouth, where they are re-chewed. Rumination constitutes a large proportion of the time budget for cattle with 6 - 7 h per day spent ruminating (Phillips, 1993), whilst for sheep approximately 3 h per day are spent ruminating (Dulphy *et al.*, 1980). The difference in the time spent ruminating may be attributed to the higher selectivity of feed by sheep and their initial chewing, which by comparison is more extensive than in cattle. Rumination is a more effective means of particle size reduction than the initial mastication due to the feedstuff being partially digested and weakened by the microbial population when it is subjected to rumination (Evans *et al.*, 1974).

The reduction in size of feed particles acts as the initial rate limiting step in digestion and passage through the rumen (Smith *et al.*, 1983; Mosely & Jones, 1984). The critical size which must be reached before particles can escape from the rumen through the reticulo-omasal orifice has been defined by Poppi *et al.* (1981b) as 1.18 mm, for both sheep and cattle. Chewing during eating reduces the particle size of feeds, releases soluble nutrients for fermentation, exposes the interior portions of the feed to microbial colonisation and hydrates dry feeds during salivation (Pond *et al.*, 1984). For forages, chewing increases ruminal degradation by increasing exposure to the potentially digestible DM and fibre fractions and by decreasing the lag time for

fibre digestion (Beauchemin *et al.*, 1994). For grains, damage to the pericarp through chewing allows microbes to gain access and colonise the nutrient - rich tissue of the endosperm (McAllister *et al.*, 1990), whilst also allowing soluble nutrients in the grain to dissolve and escape into the rumen fluid. If the whole grain is not physically damaged during chewing, digestion is severely limited (Orskov *et al.*, 1978; McAllister *et al.*, 1990). The extent of chewing required by a feed will depend upon the nature and structure of the feedstuff (McAllister *et al.*, 1990).

As many researchers involved in gas production studies also prepare feed samples by drying followed by grinding through a 1 or 4 mm dry mesh screen, the aim of this experiment was to investigate the effect of sample preparation (different drying methods and varying the size of plant particles) on the production of gas from batch cultures inoculated with rumen micro-organisms. The effect of particle size was investigated using two different types of feed, a grain and a forage, in order to determine whether the type of feedstuff influences the effect of particle size.

6.2 Materials and Methods

6.2.1 The effect of different drying treatments (Experiment 6.1)

Perennial ryegrass (*Lolium perenne*) was used as the substrate to investigate the effect of different drying procedures on gas production. The chemical composition of the ryegrass is shown in Appendix 1. The ryegrass was grown in a polytunnel under natural light conditions and watered daily.

Two days prior to the start of the experiment, a 40 g sample of grass was cut from a random plot to a stubble height of approximately 2 cm. The grass was then cut with scissors into even pieces, each approximately 3 mm long. The sample was mixed thoroughly and divided into two. Half of the sample (20 g) was dried in an oven for 48 h at 60 °C, whilst the remaining 20 g was freeze dried for 48 h.

A second 40 g sample of grass was randomly cut from the same plot 48 h after the first sample had been removed. Again the sample was cut into even pieces

approximately 3 mm long, mixed thoroughly and divided into two. One 20 g sample was kept on ice to act as the 'fresh' grass in the experiment, whilst the remaining 20 g were dried in a microwave oven (MDS 2000, Microwave Digestion System; CEM corporation, Matthews, North Carolina, USA). Microwave drying involved drying the grass sample for 6 min at 50 % power (at full power the microwave energy was 630 watts at a frequency of 2450 MHz), the drying time was interrupted after 2, 4 and 5 min of drying in order to mix the sample.

Freeze dried, oven dried, microwave dried and fresh grass (0.5 g DM) were added to 120 ml culture bottles (4 replicates per sample), containing 85 ml of warm culture medium (39 °C). After addition of the grass, each bottle was gassed with CO_2 for 3 min and 4 ml reducing agent added. The bottles were then returned to the incubator for approximately 30 min, whilst the microbial inoculum was prepared from rumen fluid collected from two rumenally-fistulated, hay-fed sheep (section 3.2.1).

Each bottle was inoculated with 10 ml of microbial inoculum, and gas production was recorded at 0, 3, 6, 9, 12, 16, 20, 24, 28, 32, 36, 44, 51, 58, 72, 96 and 120 h. At the end of the incubation pH, DM loss and VFA concentrations were determined (as described in sections 3.10, 3.10.1 and 3.12.1 respectively).

The experiment was a factorial design consisting of 4 drying treatments and 4 replicate bottles (4 x 4). The gas production profiles were fitted to the model of France *et al.* (1993) and compared using the parallel curve analysis function of MLP (Ross, 1987) (section 3.11). Values for the modelled gas production parameters, DM loss, VFA production and pH were analysed using analysis of variance in Genstat 5 (Lawes Agricultural Trust, 1993). Differences between treatments were calculated using LSD (section 3.11).

6.2.2 The effect of particle size (Experiment 6.2)

Naked oats (*Avena nuda*) and perennial ryegrass hay (*Lolium perenne*) (0.75 g) were used (Appendix 1) to investigate the effect of particle size on gas production. The

naked oats were divided into different particle sizes; whole, half, quarter, coarsely ground and finely ground by (1) manually cutting with a scalpel (half and quarter), (2) grinding in a coffee grinder (model 9701; Russell Hobbs, Failsworth, Manchester, UK) for 15 s (coarsely ground), and (3) grinding through a 1 mm dry mesh screen (finely ground).

The particle size of each treatment was calculated using Domino (Perceptive Instruments; Haverhill, Suffolk, UK). Domino uses a lens attached to a high resolution charge coupled device camera to view an illuminated field of known area. The image of any object placed on the field is digitised and displayed on the attached IBM computer monitor. The sample is detected by virtue of contrast differences and Domino calculates the area blocked out by the object. The size of the field and the measurement frame can be changed to select any particle on the field of view. The area of the image is then determined by Domino. Before particle size was measured, a calibration curve for the detection level used was calculated; particles with known area were measured using Domino, and these values were regressed against their actual area to obtain an equation for converting the area from pixels (Domino) into mm^2. The calibration factor was 0.1556 mm^2 per pixel, at a detection level of 220. The particles in the sample of naked oats which had been ground through a 1 mm mesh were too small to be quantified by Domino and hence these particles were considered to be ≤ 1 mm^2.

The sample of hay was fractionated into different particle sizes by firstly grinding the sample through a 4 mm dry mesh screen. The ground sample was then separated into 5 different sizes by wet sieving through a cascade of sieves (T and N Classifier; Turnall & Newall Ltd., Turner Asbestos Fibres, Manchester, UK). The pore size of the sieves varied from 2.4 mm^2 (at the top), through 1.2 mm^2, 0.6 mm^2 and 0.3 mm^2 to 0.15 mm^2 (at the bottom). Samples (100 g) of the ground hay were placed on the top sieve and the water supply switched on (the water cascaded through all of the sieves). When all the hay on the top sieve was soaked the machine was switched on for 2 min. The action of the machine was to rotate the sieves, whilst the contents of

each sieve (except for the lowest, 0.15 mm^2, sieve), was mixed with a paddle arm. After 2 min the machine (and the water supply) were switched off. The particles from each sieve were removed and placed in foil trays. This process was repeated until approximately 200 g of particles had been collected on each sieve. The trays were then stored at - 4 °C overnight, and freeze dried for 96 h. NSP analysis was carried out on a sample from each sieve to ensure that the chemical composition of all the particles was the same. As for the finely ground naked oats sample the majority of the hay particles were too small to be quantified by Domino and hence these particles were classified according to the size of sieve they were collected upon.

Gas production was recorded in the bottles containing naked oats at 0, 3, 6, 9, 12, 16, 20, 24, 28, 32, 36, 44, 51, 58, 72, 96 and 120 h after inoculation. Gas production in the bottles containing hay was recorded at 0, 3, 6, 9, 12, 15, 18, 21, 24, 28, 32, 37, 46, 56, 72, 96 and 145 h after inoculation. At the end of the incubation pH, DM loss and VFA concentrations were determined (as described in sections 3.10, 3.10.1 and 3.12.1 respectively).

The experiment was a factorial design consisting of 5 sizes of particle and 4 replicate bottles (5 x 4). The gas production profiles were fitted to the model of France *et al.* (1993) and compared using the parallel curve analysis function of MLP (Ross, 1987) (section 3.11). Values for the modelled gas production parameters, DM loss, VFA production and pH were analysed using analysis of variance in Genstat 5 (Lawes Agricultural Trust, 1993). Differences between treatments were calculated using LSD (section 3.11).

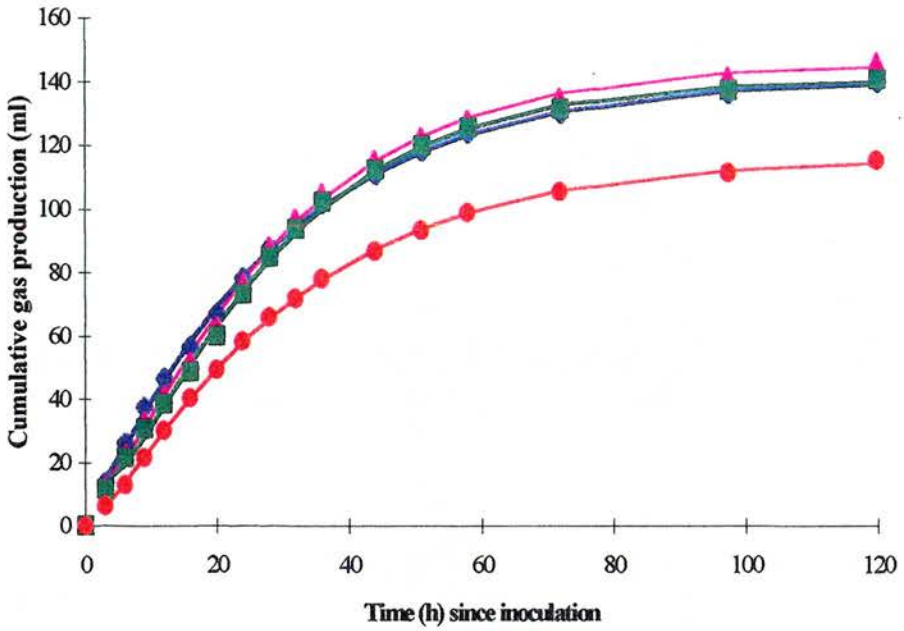
6.3 Results

6.3.1 The effect of different drying treatments (Experiment 6.1)

6.3.1.1 Gas production

Figure 6.1 shows the cumulative gas production profiles obtained during the incubation of the fresh and dried grass samples. The dried grass samples, irrespective of drying procedure, produced similar gas production profiles, whilst the fresh grass

Figure 6.1 Cumulative gas production profiles during incubation of freeze dried (-◆-), oven dried (-▲-), microwave dried (-■-) or fresh (-●-) perennial ryegrass (*Lolium perenne*) with a rumen microbial inoculum.



Each value represents the mean of four bottles, whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure. Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum.

produced lower quantities of gas than the dried samples. Parallel curve analysis indicated significant differences between the various dried samples as well as between the dried and fresh samples ($p < 0.05$; Appendix 6.1). The fitted parameters and derived quantities for the gas production profiles are shown in Table 6.1. The rates of gas production, b and c, ranged from 0.0418 h^{-1} (during incubation of the fresh sample) to 0.0573 h^{-1} (during incubation of the microwave dried sample) and from -0.1741 $h^{-0.5}$ (for the microwave dried sample) to -0.0544 $h^{-0.5}$ (for the freeze dried sample), respectively. The mean value for b for all drying treatments was 0.0486 ± 0.00383 h^{-1} whilst that for c was -0.1111 ± 0.02811 $h^{-0.5}$. Incubation of the fresh sample produced least gas (116 ml) whilst incubation of the oven dried sample produced most gas (145 ml). The mean total gas production for all drying treatments was 135.5 ± 6.69 ml. The lag time, L_T (h), encountered prior to active fermentation varied with the different drying treatments, ranging from 0.39 h (for the freeze dried sample) to 2.31 h (for the microwave dried sample), with a mean value across all drying treatments of 1.33 ± 0.445 h. The time taken to produce 50 or 95 % of the total gas production (t_{50} and t_{95}, respectively) was shortest during incubation of the freeze dried sample for t_{50} (20.5 h) and during incubation of the microwave dried sample for t_{95} (74.8 h) whilst incubation of the fresh sample produced the longest values for both t_{50} and t_{95} (24.0 and 87.2 h for t_{50} and t_{95}, respectively). The mean values for t_{50} and t_{95} were 22.3 ± 0.73 h and 80.2 ± 2.76 h, respectively.

Table 6.1 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for 0.50 g dry matter of fresh, freeze, oven or microwave dried perennial ryegrass (*Lolium perenne*) incubated with rumen micro-organisms in the pressure transducer technique.

Gas production parameters	Drying treatment				sed	Significance
	Freeze	Oven	Microwave	Fresh		
b (h^{-1})	0.0425^{a}	0.0527^{b}	0.0573^{b}	0.0418^{a}	0.0033	*
c ($h^{-0.5}$)	-0.0544^{a}	-0.1418^{b}	-0.1741^{c}	-0.0743^{a}	0.0137	*
A (ml)	140.62^{a}	145.22^{b}	140.34^{a}	115.69^{c}	1.7860	*
B	130.35^{a}	120.03^{b}	111.49^{c}	109.53^{c}	2.4130	**
L_T (h)	0.39^{a}	1.82^{b}	2.31^{c}	0.80^{d}	0.1700	*
t50 (h)	20.46^{a}	$22.29^{a,b}$	$22.57^{a,b}$	24.04^{b}	0.9710	**
t95 (h)	$81.9^{a,b}$	77.0^{a}	74.8^{a}	87.2^{b}	4.1600	*

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 6.1. Values in rows not bearing the same superscript differ significantly (*$p < 0.05$; **$p < 0.01$). The analysis of variance tables are shown in Appendix 6.2.1 - 6.2.7.

6.3.1.2 Dry matter loss

DM loss was similar for the fresh grass and grass subjected to the different drying treatments; 889.2 mg g^{-1}, 881.2 mg g^{-1}, 879.5 mg g^{-1} and 870.4 mg g^{-1} for fresh, freeze, oven and microwave dried samples respectively. However, although the values are unlikely to be significant biologically, microwave dried ryegrass was degraded to a significantly lesser extent than the fresh ryegrass ($p < 0.01$; Appendix 6.3).

6.3.1.3 Volatile fatty acid (VFA) production

VFA production for the different drying treatments are shown in Table 6.2. There were no significant differences in total VFA production between microwave, freeze or oven dried samples. However fermentation of fresh substrate produced significantly less total VFA than the dried samples ($p < 0.01$; Appendix 6.4). The

individual molar percentages of VFA showed significant differences between samples subjected to different drying treatments (Table 6.2). The percentages of acetate and propionate produced were similar between oven dried, microwave dried and fresh samples. Whilst the percentage of acetate was significantly lower ($p < 0.01$) and the percentage of propionate significantly higher ($p < 0.01$) after incubation of the freeze dried sample. The percentage of butyrate was significantly lower for the fresh sample compared to the dried samples ($p < 0.05$). The valerate percentage was similar between all drying treatments, however there was a significant difference between the oven and microwave dried samples, with a higher percentage of valerate being present after incubation of the oven dried sample ($p < 0.05$).

Table 6.2 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced from freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

	Drying Treatment					
	Freeze	Oven	Microwave	Fresh	sed	sig.
Total VFA (mmol l^{-1})	33.13[a]	34.09[a]	32.41[a]	28.10[b]	0.687	**
Acetate (molar %)	48.23[a]	51.52[b]	51.69[b]	52.31[b]	0.628	**
Propionate (molar %)	35.70[a]	32.64[b]	32.88[b]	33.03[b]	0.547	**
Butyrate (molar %)	12.69[a]	12.38[a]	12.22[a]	11.39[b]	0.343	*
Valerate (molar %)	3.48[a,b]	3.79[a]	3.21[b]	3.46[a,b]	0.149	*

Values in rows not bearing the same superscripts differ significantly (* $p < 0.05$; ** $p < 0.01$). The analysis of variance tables are shown in Appendix 6.4.1 - 6.4.5.

6.3.1.4 Changes in batch culture pH

There was no significant difference in the final pH of the culture fluid between treatments (Appendix 6.5). The mean pH value for all cultures, irrespective of the nature of the processing treatment, was 6.5 ± 0.16.

6.3.2 The effect of particle size (Experiment 6.2)

Determination of the particle sizes present in the different samples of naked oats by Domino were as follows; whole naked oats ranged from 6.46 - 15.45 mm^2, with a mean value of 11.55 ± 2.21 mm^2; half naked oats ranged in size from 4.13 - 8.78 mm^2 with a mean size of 5.99 ± 1.24 mm^2; quarter naked oats ranged from 2.05 - 5.87 mm^2 with a mean of 3.87 ± 1.13 mm^2; whilst the coarsely ground sample of naked oats ranged from 0.44 - 3.02 mm^2 with a mean of 1.27 ± 0.64 mm^2.

The hay particles were classified according to the size of the sieve they were collected upon and the range of sizes was therefore considered to be between the sieve size they remained upon and the next largest sieve; 2.4 mm^2 (ranging from 2.4 to 4 mm^2, with a geometric mean diameter (GMD) of 3.098 mm^2), 1.2 mm^2 (ranging from 1.2 to 2.4 mm^2, with a GMD of 1.697 mm^2), 0.6 mm^2 (ranging from 0.6 to 1.2 mm^2, with a GMD of 0.848 mm^2), 0.3 mm^2 (ranging from 0.3 to 0.6 mm^2, with a GMD of 0.424 mm^2) and 0.15 mm^2 (ranging from 0.15 to 0.3 mm^2, with a GMD of 0.212 mm^2). The geometric mean diameter was calculated according to the equation;

$$GMD_n = (d_n \times d_{n+1})^{0.5} \text{ (Nocek \& Kohn, 1988)} \qquad \text{(equation 6.1)}$$

where GMD_n is the geometric mean diameter of particles on the n^{th} sieve, d_n is the diameter of the pores on the n^{th} sieve and d_{n+1} is the diameter of the openings in the next larger than n^{th} sieve.

The GMD was also calculated for naked oats using the smallest and largest values for each particle size as the two sieve sizes. Hence, GMD for whole, half, quarter and coarse ground naked oats were 9.990, 6.022, 3.469 and 1.153 mm^2, respectively. These values agreed closely with the computer sized values shown above.

The composition and quantity of NSP in each of the different particle sizes of hay is shown in Table 6.3. The various particle sizes of hay contained similar quantities of rhamnose, xylose, mannose and glucose, whilst there were significant differences in

the amount of arabinose, galactose, uronic acid and hence the quantity of total NSP present. Significant differences therefore generally related to the minor NSP components, arabinose, galactose and uronic acids, which accounted for approximately 5, 2 and 3 % of the total NSP, respectively. The contribution of these minor NSP to the total NSP effectively increased the total NSP content by 7 % as the particle size decreased.

Table 6.3 Non starch polysaccharide composition (mg g^{-1} DM) of various particle sizes of hay as described in section 6.2.2.

	Particle size (mm^2)						
	2.4	1.2	0.6	0.3	0.15	s.e.d	sig.
Rhamnose	1.8	0.0	0.0	0.3	0.3	1.177	NS
Arabinose	27.0^{a}	26.9^{a}	31.8^{b}	38.3^{c}	41.2^{d}	1.178	*
Xylose	184.9	189.1	200.7	199.1	185.7	7.580	NS
Mannose	4.1	2.3	2.4	2.7	2.2	1.048	NS
Galactose	11.0^{a}	10.2^{a}	11.6^{a}	15.1^{b}	16.7^{b}	0.917	**
Glucose	345.6	355.1	383.4	383.1	364.1	16.410	NS
Uronic acid	15.2^{a}	18.5^{b}	18.7^{b}	25.4^{c}	$17.5^{a,b}$	1.068	*
Total NSP	589.6^{a}	$602.1^{a,b}$	$648.5^{b,c}$	664.1^{c}	$627.8^{a,b,c}$	22.550	*

NSP components were determined as described in section 3.12.3. Values in rows not bearing the same superscript differ significantly (* $p < 0.05$; ** $p < 0.01$). The analysis of variance tables are shown in Appendix 6.6.1 - 6.6.8.

6.3.2.1 Gas production

The cumulative gas production profiles for the naked oats of different particle sizes are shown in Figure 6.2. Half, quarter, coarse and finely ground naked oats appeared to produce similar gas production profiles, whilst gas production during incubation of whole naked oats was minimal. Although the gas production profiles looked similar for all sizes of naked oat particles which had undergone some abrasion, parallel curve analysis indicated significant differences between all profiles (the rate of gas production between quarter and coarse naked oats being the only exception) ($p < 0.01$; Appendix 6.7).

Figure 6.2 Cumulative gas production profiles obtained during incubation of whole (-◆-), half (-▲-), quarter (-■-), coarse ground (-×-) and ≥ 1 mm (-●-) naked oats (*Avena nuda*) with a rumen microbial inoculum.

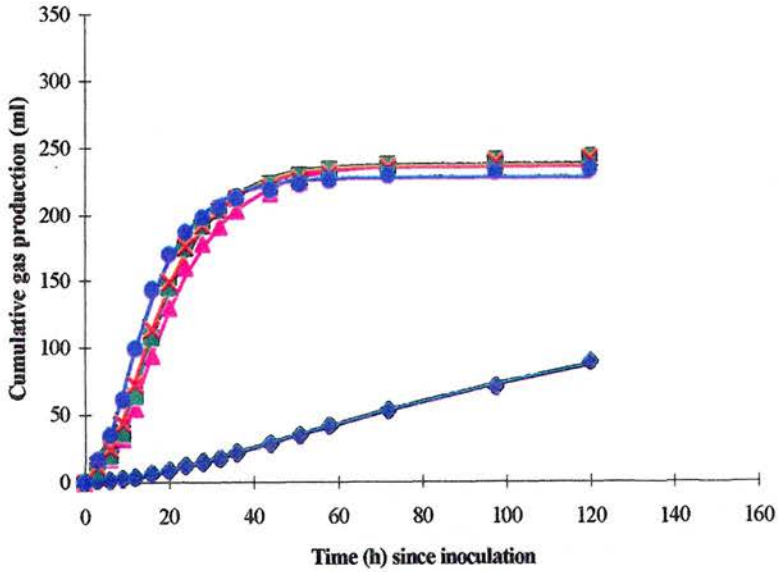
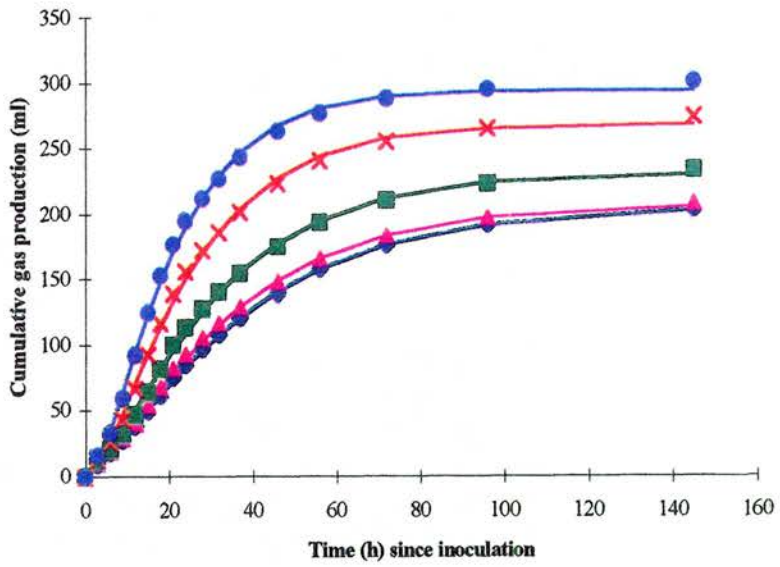


Figure 6.3 Cumulative gas production profiles obtained during incubation of perennial ryegrass (*Lolium perenne*) hay particles with a size of 2.4 (-◆-), 1.2 (-▲-), 0.6 (-■-), 0.3 (-×-) and 0.15 mm (-●-) with a rumen microbial inoculum.



Each value represents the mean of four bottles, whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure. Fermentations were conducted in 160 ml serum bottles containing 89 ml of culture medium and 10 ml of rumen microbial inoculum. The various particle sizes were obtained as described in section 6.2.

The fitted parameters and derived quantities for the gas production profiles are shown in Table 6.4. The rates of gas production, b and c, ranged from 0.0100 h^{-1} (during incubation of the whole naked oats) to 0.1566 h^{-1} (during incubation of the finely ground naked oats) and from -0.5965 $h^{-0.5}$ (for the quarter naked oats) to -0.0462 $h^{-0.5}$ (for the whole naked oats), respectively. The mean value for b for all sizes of particle was 0.1155 ± 0.02696 h^{-1} whilst that for c was -0.4392 ± 0.09967 $h^{-0.5}$. Incubation of the whole naked oats produced least gas (158 ml) whilst incubation of the half, quarter, coarsely and finely ground naked oats produced similar quantities of gas (228 - 238 ml). The mean total gas production for all sizes of naked oat was 218.4 ± 15.18 ml. The lag time, L_T (h), encountered prior to active fermentation ranged from 2.76 h (for the finely ground naked oats) to 5.46 h (for the whole naked oats), with a mean value across all sizes of particle of 4.01 ± 0.435 h. The time taken to produce 50 or 95 % of the total gas production (t_{50} and t_{95}, respectively) was shortest during incubation of the finely ground naked oats (13.4 and 35.8 h for t_{50} and t_{95}, respectively) whilst incubation of the whole naked oats produced the longest values for both t_{50} and t_{95} (31.1 and 202.4 h for t_{50} and t_{95}, respectively). The mean values for t_{50} and t_{95} were 19.3 ± 3.06 h and 74.1 ± 32.14 h, respectively. The large value of t_{95} obtained for the whole naked oats is likely to be due to the lack of an asymptote in their gas production profile; gas production slowly increasing until the end of the incubation.

Table 6.4 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for 0.75 g naked oats of different particle sizes incubated with rumen micro-organisms in the pressure transducer technique.

Gas production parameters	Particle size					sed	sig
	Whole	Half	Quarter	Coarse	Fine		
b (h^{-1})	0.0100^{a}	0.1229^{b}	0.1475^{c}	0.1407^{c}	0.1566^{d}	0.003	*
c ($h^{-0.5}$)	-0.0462^{a}	-0.4947^{b}	-0.5965^{c}	-0.5391^{d}	$-0.5195^{b,d}$	0.017	*
A (ml)	158.1^{a}	231.4^{b}	237.6^{b}	236.8^{b}	228.2^{b}	7.240	***
B	149.2^{a}	139.9^{a}	125.8^{b}	141.5^{a}	$138.6^{a,b}$	6.070	*
L_T (h)	5.46^{a}	4.05^{b}	4.09^{b}	$3.67^{b,c}$	2.76^{c}	0.448	*
t50 (h)	31.08^{a}	18.73^{b}	$17.12^{b,c}$	16.39^{c}	13.40^{d}	0.759	*
t95 (h)	202.45^{a}	47.93^{b}	42.31^{c}	42.14^{c}	35.84^{d}	0.868	***

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 6.2. Values in rows not bearing the same superscript differ significantly (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The analysis of variance tables are shown in Appendix 6.8.1 - 6.8.7.

The cumulative gas production profiles for the different sizes of hay particles are shown in Figure 6.3. Throughout the incubation the largest volumes of gas were produced in bottles containing particles from the 0.15 mm^2 sieve, with smaller volumes of gas being produced by particles collected on the 0.30, 0.60 and 1.20 mm^2 sieves in turn and least gas being produced by particles from the 2.4 mm^2 sieve. Parallel curve analysis of the gas production profiles from the different particle sizes of hay incubated showed significant differences between both the rate and total cumulative volume of gas produced for all particles ($p < 0.001$; Appendix 6.9). The fitted parameters and derived quantities for the gas production profiles are shown in Table 6.5. The rates of gas production, b and c, ranged from 0.0365 h^{-1} (during incubation of particles from the 2.4 mm^2 sieve) to 0.0807 h^{-1} (during incubation of particles from the 0.15 mm^2 sieve) and from -0.2189 $h^{-0.5}$ (for particles from the 0.15 mm^2 sieve) to -0.0942 $h^{-0.5}$ (for particles from the 2.4 mm^2 sieve), respectively. The

mean value for b for all sizes of particle was 0.0543 ± 0.00809 h^{-1} whilst that for c was -0.1475 ± 0.02278 $h^{-0.5}$. Incubation of particles from the 2.4 mm^2 sieve produced least gas (207 ml) whilst incubation of particles from the 0.15 mm^2 sieve produced most gas (295 ml). The mean total gas production for all sizes of particle was 242.4 ± 17.29 ml. The lag time, L_T (h), encountered prior to active fermentation was similar between the different sizes of particle, ranging from 1.67 h (for particles from the 2.4 mm^2 sieve) to 2.06 h (for particles from the 0.6 mm^2 sieve), with a mean value across all sizes of particle of 1.84 ± 0.068 h. The time taken to produce 50 or 95 % of the total gas production (t_{50} and t_{95}, respectively) was shortest during incubation of particles from the 0.15 mm^2 sieve (17.8 and 55.1 h for t_{50} and t_{95}, respectively) whilst incubation of particles from the 2.4 mm^2 sieve produced the longest values for both t_{50} and t_{95} (30.5 and 105.9 h for t_{50} and t_{95}, respectively). The mean values for t_{50} and t_{95} were 24.7 ± 2.31 h and 81.8 ± 9.33 h, respectively.

Table 6.5 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for 0.75 g hay of different particle sizes incubated with rumen micro-organisms in the pressure transducer technique.

Gas production parameters	Particle size (mm^2) 2.4	1.2	0.6	0.3	0.15	sed	sig
b (h^{-1})	0.0365^a	0.0408^b	0.0495^c	0.0638^d	0.0807^e	0.001	**
c ($h^{-0.5}$)	-0.0942^a	-0.1071^a	-0.1417^b	-0.1754^c	-0.2189^d	0.007	***
A (ml)	206.6^a	209.4^a	231.7^b	269.4^c	295.0^d	6.570	**
B	187.2^a	186.8^a	201.0^a	231.4^b	246.4^c	6.690	*
L_T (h)	1.67^a	$1.72^{a,b}$	2.06^b	$1.89^{a,b}$	$1.84^{a,b}$	0.172	*
t50 (h)	30.51^a	28.23^b	25.69^c	21.14^d	17.82^e	0.543	**
t95 (h)	105.94^a	96.86^b	84.03^c	67.20^d	55.06^e	1.700	***

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 6.3. Values in rows not bearing the same superscript differ significantly (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The analysis of variance tables are shown in Appendix 6.10.1 - 6.10.7.

6.3.2.2 Dry matter loss

DM loss was significantly lower for whole naked oats (344.5 mg g^{-1}) compared to all other sizes of particles tested; 929.9 mg g^{-1}, 957.4 mg g^{-1}, 904.5 mg g^{-1} and 957.5 mg g^{-1} for half, quarter, coarsely ground and finely ground particles, respectively ($p < 0.001$; Appendix 6.11). With cut and ground samples of naked oats, DM loss was not significantly different.

DM loss for hay was significantly lower during incubation of the larger particle sizes (729.8 mg g^{-1}; 723.2 mg g^{-1} and 724.1 mg g^{-1}, for particles from the 2.4, 1.2 and 0.6 mm^2 sieves respectively) compared to the smaller particle sizes (751.9 mg g^{-1} and 753.6 mg g^{-1} for particles from the 0.3 and 0.15 mm^2 sieves respectively) ($p < 0.001$; Appendix 6.12).

6.3.2.3 Volatile fatty acid (VFA) production

Production of VFA for the different particle sizes of naked oats are shown in Table 6.6. Total VFA production was significantly greater from half, quarter, coarse and finely ground naked oats compared to whole naked oats ($p < 0.01$; Appendix 6.13). There were also differences in the VFA molar percentages (Table 6.6). Whole naked oats resulted in a significantly lower percentage of acetate ($p < 0.05$) and butyrate ($p < 0.01$), but a higher percentage of propionate ($p < 0.01$) and valerate ($p < 0.01$) than all other particle sizes. The highest percentage of acetate was produced during incubation of coarse and finely ground naked oats ($p < 0.05$). The percentage of propionate was significantly higher after incubation of whole naked oats compared to half naked oats ($p < 0.01$) which in turn resulted in a higher percentage of propionate than incubation with quarter and coarse naked oats ($p < 0.01$). Incubation of coarsely ground naked oats resulted in the lowest percentage of propionate. Incubation of half, quarter and coarse naked oats resulted in a significantly higher percentage of butyrate than incubation of finely ground naked oats ($p < 0.01$), which in turn produced a significantly higher percentage of butyrate than whole naked oats ($p < 0.01$). The percentage of valerate after incubation was similar for all particle sizes, with the

exception of whole naked oats were the percentage of valerate was significantly higher ($p < 0.01$).

Table 6.6 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced from different particle sizes of naked oats during incubation with a rumen microbial inoculum.

	Particle size					
	Whole	Half	Quarter	Coarse	Fine	sed
Total VFA (mmol l^{-1})	20.02^{a}	49.39^{b}	49.44^{b}	51.35^{b}	47.81^{b}	1.289
Acetate (molar %)	35.32^{a}	39.59^{b}	43.99^{c}	$45.42^{c,d}$	46.47^{d}	0.858
Propionate (molar %)	38.63^{a}	25.69^{b}	20.14^{c}	19.38^{c}	21.46^{d}	0.419
Butyrate (molar %)	18.96^{a}	30.20^{b}	31.72^{b}	30.44^{b}	27.26^{c}	0.730
Valerate (molar %)	7.61^{a}	4.52^{b}	4.15^{b}	4.76^{b}	4.80^{b}	0.652

Values in rows not bearing the same superscripts differ significantly ($p < 0.01$). With the exception of the values for acetate where $p < 0.05$. The analysis of variance tables are shown in Appendix 6.13.1 - 6.13.5.

Volatile fatty acid (VFA) production for the different sizes of hay particles are shown in Table 6.7. Total VFA production was similar after incubation involving hay particles from the 1.2, 0.6, 0.3 and 0.15 mm^2 sieves. These particles all tended to produce more VFA than incubations involving particles from the 2.4 mm^2 sieve. However, only incubation with particles from the 1.2 and 0.6 mm^2 sieves produced significantly more VFA than incubation with particles from the 2.4 mm^2 sieve ($p < 0.05$). The molar percentages of VFA were also seen to vary according to the different sizes of hay particles (Table 6.7). The percentage of acetate produced was similar for all sizes of particles. However acetate production from the 1.2 and 0.6 mm^2 sieve particles was significantly lower than the acetate production from particles from the 0.15 mm^2 sieve ($p < 0.05$). The percentage of propionate produced was similar between particles from the 2.4, 1.2 and 0.6 mm^2 sieves. Incubation with

particles from the 0.3 mm^2 sieve resulted in a significantly lower percentage of propionate than that produced with particles from the 0.6 mm^2 sieve ($p < 0.05$), whilst incubation of particles from the 0.15 mm^2 sieve resulted in a significantly lower percentage of propionate than incubation of particles from the 2.4, 1.2 and 0.6 mm^2 sieves ($p < 0.05$). There were no significant differences in the percentage of butyrate or valerate produced after incubation of the different sizes of hay particles.

Table 6.7 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced from different particle sizes of perennial ryegrass hay (*Lolium perenne*) during incubation with a rumen microbial inoculum.

	Particle size (mm^2)					
	2.4	1.2	0.6	0.3	0.15	sed
Total VFA (mmol l^{-1})	43.80^{a}	53.20^{b}	53.40^{b}	$48.90^{a,b}$	$48.90^{a,b}$	3.220
Acetate (molar %)	$62.25^{a,b}$	61.00^{a}	61.15^{a}	$61.96^{a,b}$	63.46^{b}	0.885
Propionate (molar %)	$29.44^{a,b}$	$29.83^{a,b}$	30.09^{a}	$28.89^{b,c}$	28.10^{c}	0.492
Butyrate (molar %)	6.51	7.16	7.00	6.92	6.81	0.352
Valerate (molar %)	1.79	2.01	1.76	2.24	1.63	0.311

Values in rows not bearing the same superscripts differ significantly ($p < 0.05$). The analysis of variance tables are shown in Appendix 6.14.1 - 6.14.5.

6.3.2.4 Changes in batch culture pH

The pH after incubation of different particle sizes of naked oats was significantly higher for whole naked oats (pH 6.5) compared to all the smaller particle sizes ($p < 0.001$; Appendix 6.15). The pH was similar after incubation of halved, quartered, coarsely ground and finely ground naked oats, with a mean pH of 6.3 ± 0.01.

The pH after incubation of different particle sizes of hay was not significantly different between the different sizes of particles (Appendix 6.16). The mean pH value for all sizes of hay particles was 6.5 ± 0.10.

6.4 Discussion

The lower gas and VFA production for the fresh grass compared to all drying treatments suggests that drying alters the plant cells making them easier to degrade. However, the resulting DM degradation values were not consistent with this hypothesis as samples of fresh grass appeared to be degraded to a similar extent as the freeze dried and oven dried samples, whilst being degraded to a significantly greater extent than the microwave dried samples. One explanation for this discrepancy between fresh and dried samples could be related to microbial contamination. The mechanism of tissue degradation by rumen micro-organisms varies for different feeds and with the different physical structures of plant cell components (Olubobokun *et al.*, 1990); rumen micro-organisms attaching preferentially to exposed, cut or damaged tissues (Latham *et al.,* 1978; Chesson & Forsberg, 1997). Latham *et al.* (1978) reported that both *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* were preferentially bound to cut or damaged plant cell wall surfaces of fresh ryegrass during its digestion. *Fibrobacter succinogenes* adhered to the cut edges of most plant cells except those of xylem, as well as adhering to the uncut surfaces of mesophyll, epidermis and phloem cell walls, whilst *Ruminococcus flavefaciens* predominated on uncut surfaces of epidermis, phloem and schlerenchyma cell walls. The different drying processes may have altered or damaged the ryegrass cells in such a way as to increase microbial attachment. For example, drying may have damaged the waxy cuticle of the grass (which is inert to the rumen microbes) making microbial colonisation easier (Chesson & Forsberg, 1997). A considerable proportion of the micro-organisms which attach to ingested feed particles (and to *in vitro* residues) remain firmly attached even after extensive washing (Olubobokun & Craig, 1990), hence residues will contain microbial DM in addition to the DM of the feed residue. As microbial contamination is not accounted for during the measurement of DM loss, higher microbial contamination of the dried samples may have resulted in the apparently similar DM loss to the fresh ryegrass which may have had less microbial contamination. Alternatively, this difference may be explained by the efficiency of the micro-organisms in the presence of fresh and dried herbage. In the presence of fresh herbage the proteolytic activity of rumen

contents has been reported to be up to nine times greater than that found with dry rations (as the higher soluble-protein content of the fresh herbage enriches for proteolytic bacteria; Nugent & Mangan, 1981; Nugent *et al.*, 1983). Degradation of protein in the fresh grass may also have been aided by the presence of endogenous plant proteases (Theodorou, 1995). If these plant proteases were destroyed by the drying treatments employed in this study, this could explain the similar DM loss but lower VFA production during incubation of the fresh grass compared to all dried samples.

All forms of drying that involve heat will result in some change in composition, physical state or solubility of the feed (Jones & Moseley, 1993). For example, proteins are denatured at high temperatures and linkages may form between carboxyl groups of sugars and the amino acids of proteins to form Maillard compounds, which have physical properties similar to lignin (Van Soest, 1982; Van Soest & Mason, 1991). Prolonged oven drying at 100 °C has been shown to reduce *in vitro* digestibility of forage samples (Tilley & Terry, 1963). Drying of fermented feeds, such as silage, may lead to volatile losses of alcohols, acids, esters, amines and ammonia hence decreasing the nutritional value of the feed (Snyman & Joubert, 1992; Murray, 1993). Oven drying (60 °C) has also been shown to affect the estimation of both fibrous and water soluble carbohydrate fractions of maize silage and analysis on wet samples is recommended (Piccaglia & Galletti, 1987).

Lopez *et al.* (1995) measured higher *in situ* DMD with freeze and oven dried samples of ryegrass and white clover, compared to that of fresh samples. They hypothesised that these differences could be due to differences in the particle size and physical form induced by grinding rather than to the drying process itself. However, Uden (unpublished data, 1990; discussed in Uden, 1992) suggests that the vegetative parts of plants are protected from microbial attack while the plant is alive. Three week old whole green barley leaves being significantly more resistant to microbial attack at 9 h incubation time *in situ* than when dried. Changes induced during the drying processes may therefore result in disruption of the plants barriers to microbial fermentation.

In vitro organic matter digestibility (OMD) of forage samples has also been shown to be affected by drying treatment, the OMD of oesophageal fistula samples which were oven dried (40 °C) being depressed more than air or freeze dried samples (Burritt *et al.*, 1988). Broesder *et al.* (1992) observed differences in the *in vitro* OMD of masticated forage samples subjected to freeze or oven drying (60 °C), although the rates of OM and NDF degradation were not affected. Kamoun and Thewis (1990) also reported lower values for *in vitro* OMD of oven dried compared to freeze dried grass, whilst Karn (1991) found that heat drying (microwave or oven [50 °C]) had little effect on *in vitro* OMD of forages compared with freeze drying. However, NDF was apparently increased in the microwave and oven dried samples. Although freeze drying is preferable (Abdalla *et al.*, 1988; Burritt *et al.*, 1988; Lopez *et al.*, 1995), oven drying at low temperatures and microwave drying are also suitable for preparation of samples for *in situ* studies (Chamberlain & Endalew, 1993). For example, Kabuga and Darko (1993) reported that both DM and nitrogen degradabilities of oven dried (45 °C) and fresh samples were similar throughout the *in situ* incubation of four tropical grasses. However, if fibre levels were to be determined samples should be freeze dried for the most accurate results (Karn, 1991).

Smith (1973) reviewed the influence of drying methods on the content of non-structural carbohydrates of herbage and concluded that freeze-drying gave results closest to those found for fresh herbage. Heat drying below 80 °C was satisfactory for the estimation of total water-soluble carbohydrates, although freeze-drying was recommended for analysis of constituent sugars.

Decreasing particle size tended to increase both the rate and final volume of gas produced, and correspondingly DM loss was increased in both naked oats and hay. The most likely explanation for this phenomenon is that the decrease in particle size led to an increase in the surface area which increased the area available to microbial attack (Emanuele & Staples, 1988; Bowman & Firkins, 1993; Chesson, 1993).

During incubation of the naked oats, all particle sizes produced similar gas profiles and values of DM loss with the exception of the whole grain where gas production was approximately 32 % lower and DM loss was 63 % lower than that seen with the smaller particles. These findings agree well with those of McAllister *et al.* (1990) where whole, halved and quartered cereal grains were incubated *in situ*, resulting in significantly lower DMD of whole grains ($p < 0.001$) compared to halved and quartered grains (which had similar *in situ* DMD). This may be explained by the physical structure of the whole grains. Seeds often have impenetrable cell walls and cutin layers to survive passage through the digestive tract and make seed dispersal by animals possible. Grains are generally protected by the pericarp which acts as a barrier to microbial digestion of the underlying tissues. Scanning electron microscopy has demonstrated the extreme resistance to bacterial colonisation of the outside surface of wheat, barley and maize kernels, the kernels being almost devoid of bacteria throughout a 48 h incubation *in situ* (McAllister *et al.*, 1990). However, when the pericarp was damaged by cutting or grinding the highly soluble, nutrient-rich endosperm became available to the micro-organisms, greatly increasing both the rate and extent of degradation. Damage to the pericarp allows both the soluble constituent to escape into the rumen fluid and bacteria to gain access to and colonise the nutrient-rich endosperm, which is essential for the digestion of cereal grains in the rumen (McAllister *et al.*, 1990). The attachment of rumen micro-organisms occurring preferentially to exposed, cut or damaged tissue (Olubobokun *et al.*, 1990). Damage to the pericarp also allows soluble material to escape, with soluble protein eliciting the attachment of protozoa to plant particles (Orpin & Letcher, 1978), hence increasing digestion. These results agree well with the findings of Beauchemin *et al.* (1994), where less than 30 % of the DM disappeared from whole grains (barley, corn and wheat) incubated *in situ* for 96 h, and the digestion kinetics were characterised by a negligible soluble fraction, a lag phase and a slow fractional rate of disappearance of the potentially digestible fraction. The DM loss was greatly increased when the grains were damaged by ingestive mastication or by manually sectioning the grains into halves or quarters before incubating in the rumen.

The gas production profiles obtained for the hay were more diverse than those seen for the naked oats; whereas naked oats produced similar gas production profiles for all particle sizes except whole naked oats, gas production from the different sizes of hay particles tended to increase with the decrease in particle size. This supports the hypothesis that an increase in surface area leads to an increase in digestion due to the larger surface area available for microbial attachment. Correspondingly, Davies (1991) found similar gas production profiles to those obtained for hay during the incubation of different particle sizes of straw. The NSP composition differed between the various particle sizes of hay for arabinose, galactose, uronic acid and hence the total quantity of total NSP, with the smaller particles generally containing more of these components than the larger particles. This difference in chemical composition may be influenced by fragmentation phenomenon, for example, smaller particles may be small because of their chemical and physical configuration and they therefore fragment into small particles when ground, whilst the chemical configuration of other particles may favour fragmentation into larger particles. Longland *et al.*, (1995) found that in eleven samples of tropical forage legume the most degradable NSP constituent was the uronic acids followed by arabinose, whilst glucose and xylose were least degradable. Therefore the increased quantities of uronic acids and arabinose in the smaller particles should have been easily degraded. In addition loss of uronic acids from the smaller particles may have been greater than that from the large particles, as physical damage which allows pectolytic rumen bacteria to colonise the pectic fraction has been reported to be more important than the intrinsic chemical nature of the uronic acids found in legumes (Chesson & Munro, 1982).

Increase in the rate and extent of digestion in the rumen with decreasing particle size has also been observed *in situ*. For example, Bowman and Firkins (1993) reported that the *in situ* rates of NDF and ADF disappearance were faster for forages ground through a 2 mm screen compared to those ground to pass a 5 mm screen. However, Weakley *et al.* (1983) found that pulverising soyabean meal or distillers grains for 8 h resulted in greater *in situ* DM and N digestibilities than when soyabean meal in its commercially processed form, or distillers grains ground through a 2 mm mesh

screen were incubated. However the effect of reducing particle size on *in situ* digestion does not appear to be uniform between forages (Emanuele and Staples, 1988) nor between non-forage feeds (Ehle *et al.*, 1982a). For example, Robles *et al.* (1980) found that digestion rate constants for orchardgrass did not differ among samples ground through 1, 4, 8 or 12 mm screens, whilst alfalfa stems ground through 1 or 4 mm screens had rate constants twice that of alfalfa stems ground through 8 or 12 mm screens. Differences in the effect of particle size between forages may be due to chemical and / or structural differences between the forages. For example, Akin (1980) hypothesised that loosely structured cell walls did not require microbial attachment for digestion, extracellular enzymes being able to degrade the cell walls, whilst highly structured cell walls required bacterial attachment in order for degradation to occur. Therefore the easily degraded substrates may be degraded more quickly by increasing surface area as there will be a larger surface area for extracellular enzymes to attack whilst less degradable substrates may still require microbial attachment in order to degrade the particles. Hence, a simple reduction in particle size does not necessarily increase digestibility or the rate of digestion if the small particles still contain a high proportion of poorly degradable material, such as lignin (Pond *et al.*, 1984). Olubobokun *et al.* (1990) suggested that the removal of soluble nutrients due to mastication or grinding may be more important to microbial attachment and increased digestibility than exposed surface area. Thus feeds with a high soluble content, such as naked oats, would become more degradable whilst feeds with little soluble material, such as hay, may be less affected by a reduction in particle size.

The effect of reducing the particle size of feedstuffs has also been investigated *in vivo*. Firkins *et al.* (1986) measured higher digestibilities for OM and NDF in the rumen of steers fed ground prairie grass hay (10 mm screen) compared to those fed chopped grass hay (50.8 mm screen). Since there were no significant differences in ruminal particulate dilution rate (both ground and chopped hay particles being retained in the rumen for the same length of time) the increase in digestibility was attributed to the greater surface area per g of DM of ground hay allowing more

colonisation by the ruminal microbial population and subsequently, more extensive fermentation of ground versus chopped hay. However, reducing the particle size of hay by chopping has also been seen to decrease digestibility *in vivo*, by increasing the rate of passage through the rumen (Jaster & Murphy, 1983). The increase in digestion rate when small particles are fed, may not have occurred in the study of Firkins *et al.* (1986) due to the small difference in hay particle size, 10 mm versus 50.8 mm, whilst Jaster and Murphy (1983), used a wider size range; long hay (no measurement of the actual size was given however the long hay sample consisted of grass which was mown and collected without any chopping) versus finely chopped hay approximately 2.3 mm long, accentuating the reduction in particle size. Stratification of the rumen may also affect the passage rate of particles through the rumen. Large particles may become entangled within the 'raft' of plant particles which floats on the liquid digesta maintained by the entrapment of fermentation gas within the plant particles. Whilst small particles, which would otherwise pass from the rumen through the reticulo-omasal orifice, may also become entrapped in the raft, hence reducing passage rate (Wilson & Kennedy, 1996).

Passage rate through the rumen plays an important role in digestion *in vivo*, which is not simulated by batch cultures. The extent of ruminal digestion is therefore influenced by ruminal passage rate which is affected by feed particle size, with dilution rate being negatively correlated to digestion (i.e. as dilution rate increases, digestion decreases). Differences in the effect of particle size on digestibility *in vivo* may be due to the physical structure of the resultant ground material, thin, long, needle-like shapes passing through the rumen more slowly than short, fat, oblong shapes (Emanuele & Staples, 1988), hence needle-like shapes are unlikely to increase the rate of passage resulting in increased digestibility with decreasing size. The functional specific gravity and buoyancy of the particles will also affect the rate of passage; small particles with a low functional specific gravity or high buoyancy are likely to leave the rumen at a slower rate than particles of the same size which have a higher functional specific gravity or lower buoyancy (McLeod *et al.*, 1990; Wilson & Kennedy, 1996).

Reducing the variability of particle size by grinding through a 1 mm mesh may not mimic the *in vivo* conditions ideally but it does tend to increase the precision of both *in vitro* and *in situ* measures (Johnson, 1966; Alexander, 1969; Nocek, 1988; Weiss, 1994). For example, Mehrez and Orskov (1977) found that when rolled barley was incubated *in situ* there was a wide range in DMD from different bags, when the contents of the bags were inspected more whole grains were seen in the bags with a low percentage DMD compared to those bags with high DMD. The variability was greatly reduced when whole grains were discarded before the incubation. In addition the particle shapes which were produced as a result of mechanical grinding appear to be similar to those produced from chewing and digestion (Emanuele and Staples, 1988).

Particle size is an important factor affecting the rate of fermentation and hence influences rumen function and ultimately animal performance, for example, milk fat production (Cherney *et al.*, 1988). Although reduction of particle size generally increases the rate of fermentation, Lindberg (1981) noted that the influence of grinding was greatest when the incubation time was short, small particles can pass unfermented from the rumen. If this passage rate exceeds the increase in fermentation rate, the overall extent of rumen fermentation can be reduced and the total animal digestion becomes more dependent upon postruminal enzymatic and fermentative digestion (Russell & Hespell, 1981). Particles with a mean size of 0.2 mm are likely to escape from the rumen of sheep with essentially no reduction in size (Smith *et al.*, 1983), whereas particles which are greater than 1.18 mm have a high resistance to passage from the sheep rumen and therefore generally require size reduction by rumination (Poppi *et al.*, 1980a & b).

Gerson *et al.* (1988) and Uden (1992) investigated the effect of particle size on fermentation using *in vitro* techniques in order to minimise the problems of loss or gain of fine particles. Gerson *et al.* (1988) showed that external particle surface area cannot be used directly as an index of fermentation rate. Gas production was only 30 % lower for the large particles (2 mm^2) in spite of the surface area being 10 % that of

the small particles. Thus they concluded that the rate of gas production depends upon the number of bacteria adhering to particles, population density increasing with increased surface area. Uden (1992) concluded that the lag of fermentation is more affected by particle size than the rate. Beauchemin (1992) also concluded that chewing during eating increased rumen degradation of forage by increasing the potentially digestible DM fractions and by decreasing the lag time for fibre digestion, but not by altering the rate of digestion. However, the rate of degradation of a large particle in the rumen may alter as particle size is reduced and this should be taken into consideration when modelling the degradation process (Bowman *et al.*, 1991).

This study has shown that sample preparation is an important factor in gas production studies. The differences between the gas production profiles for fresh and dried samples of grass suggest a change in microbial efficiency, hence substrates should, if possible, be incubated in the state in which they are to be fed. In addition particle size had a significant impact on gas production profiles. A large gradation in gas production was seen with the hay particles; more gas being produced as particle size decreased. Particle size also affected the gas production profiles from naked oats; whole naked oats producing significantly less gas than those which had undergone some form of abrasion. This suggests that for highly soluble feedstuffs particle size is of little importance as long as the feed has undergone some form of abrasion, whilst for more fibrous, less soluble feedstuffs particle size is more important. Extrapolating the particle size data from gas production studies to the animal is not possible due to the wide range of feed particle sizes which are found in the animal. More importantly these particles are continually changing shape and size as they are masticated and fermented by the animal and its micro-organisms (Weston & Kennedy, 1984). The particle size of feedstuffs also affects their rate of passage through the rumen and this could not be simulated via the pressure transducer technique, due to its batch culture nature. The *in vitro* gas production technique is therefore unlikely to simulate digestion *in vivo* exactly, however, drying and grinding feeds before incubating reduces variation (increasing precision) and may produce particle shapes similar to

those produced through mastication and rumination (Emanuele & Staples, 1988; Michalet-Doreau & Ould-Bah, 1992).

CHAPTER 7 - A COMPARISON BETWEEN TWO GAS PRODUCTION TECHNIQUES FOR ESTIMATING THE FERMENTATION PARAMETERS OF THREE FEEDSTUFFS

7.1 Introduction

As discussed in chapter 2, gas production has become a popular tool for characterising feeds and investigating the kinetics of rumen fermentation. Since the method was first described by Menke *et al.* in 1979 several new techniques have been developed for recording the production of gas during the fermentation of a feedstuff. For example, Beuvink *et al.* (1992), Pell & Schofield (1993), Theodorou *et al.* (1994), Davies *et al.* (1995) and Cone *et al.* (1996). The main principle of all these techniques is the same; i.e. electronic measurement of gas production during the incubation of a feed sample with rumen fluid. However the methods employed differ as described in section 2.1.4.3.

In order for the gas production method to be of value as a tool for feed evaluation the procedure will need to be standardised. Standardisation was an important step in the development of the Tilley and Terry (1963) technique, permitting reproducible results between laboratories. For example the composition of the medium, incubation time and the time and method of collection of rumen fluid from the donor animals were all standardised. At present, the composition of the medium, time and method of rumen fluid collection, diet of the donor fistulated animal and the technique itself vary between the different laboratories which use these gas production techniques. The culture medium (buffer) to rumen fluid ratio also differs dramatically; from 2:1 (Menke *et al.*, 1979) to 9:1 (Theodorou *et al.*, 1994) in these gas production procedures.

The aim of this study was therefore to compare the gas production profiles, DM loss and VFA production for three diverse feedstuffs; naked oats (*Avena nuda*), oatfeed and ryegrass (*Lolium multiflorum*), using the two most commonly used manual techniques; the Menke *et al.* (1979) technique (MT) and the pressure transducer

technique (PTT) (Theodorou *et al.*, 1994), using different medium : rumen fluid ratios (M:RF) and keeping everything constant between the two techniques. The M:RF ratios chosen were as follows; (1) 2:1 - the ratio associated with the Menke technique (2) 9:1 - the ratio associated with the PTT and (3) 6:1 - a 'middle' value.

7.2 Materials and Methods

Three feeds, naked oats (NO), oatfeed (OF) and ryegrass (*Lolium multiflorum*) (RG) were used. All feeds were ground to pass through a 1mm mesh screen. The composition of the feedstuffs are shown in Appendix 1.

The medium described by Menke & Steingass (1988) was used in both the pressure transducer and Menke techniques. The medium was composed of 474 ml distilled water, 0.12 ml trace element solution, 237 ml buffer solution, 237 ml main element solution and 1.22 ml resazurin solution; the composition of each solution is shown in Table 7.1. The medium (950 ml) was reduced by the addition of 50 ml of freshly prepared reducing agent (Menke & Steingass, 1988).

The microbial inoculum was prepared from bovine rumen digesta (section 3.2.2) by straining the digesta through 3 layers of muslin. In accordance with the Menke technique, the rumen fluid was not macerated before being strained.

The Pressure Transducer Technique (PTT)

Naked oats, oatfeed or ryegrass (300 mg) were weighed out in triplicate into 70 ml serum bottles and 27, 34 or 36 ml of culture medium added. Sealed bottles were left at room temperature for 12 h, warmed to 39 °C and inoculated with either 13, 6 or 4 ml of freshly prepared microbial inoculum, to give a total volume (medium + rumen fluid) of 40 ml per bottle and M:RF ratios of 2:1, 6:1, and 9:1. Bottles were zeroed (section 3.9) and incubated at 39 °C. Gas production was recorded at 2, 4, 6, 8, 10, 12, 15, 18, 21, 24, 28, 32, 36, 44, 51, 58, 72, 96 and 144 h after inoculation.

Table 7.1 The composition of the solutions used to prepare the culture medium, as described by Menke & Steingass (1988).

Solution	g
Main element solution:[1]	
Na_2HPO_4	5.7
KH_2PO_4	6.2
$MgSO_4.7H_2O$	0.6
Buffer solution:[1]	
$NaHCO_3$	35.0
$(NH_4)HCO_3$	4.0
Trace element solution:[2]	
$CaCl_2.2H_2O$	13.2
$MnCl_2.4H_2O$	10.0
$CoCl_2.6H_2O$	1.0
$FeCl_2.6H_2O$	0.8
Resazurin solution:[2]	
Resazurin	0.1
Reduction solution:[3]	
1 - n - NaOH	2 ml
$Na_2S.7H_2O$	285 mg

[1] made up to 1 litre with distilled water
[2] made up to 100 ml with distilled water
[3] added to 47.5 ml distilled water

The Menke Technique

Gas production was measured according to the method of Menke and Steingass (1988). Naked oats, oatfeed or ryegrass (300 mg) were weighed into 100 ml, graduated, syringes and 40 ml of culture medium / rumen fluid mixture added by pipette; as for the PTT, three different M:RF ratios were used; 2:1, 6:1, and 9:1. The

culture medium was mixed with the rumen fluid, in the appropriate ratios, before being added to the syringes. This was done under CO_2 gas, in a water bath (39 °C). The syringes were sealed by attaching a short silicone tube (approximately 5 cm long) to the syringe's capillary attachment and closing the open end by the use of a small clamp. The volume was read from the calibration on the syringe. The syringe was then placed in a water bath at 39 °C. Gas production was recorded throughout the incubation by noting the place of the piston in the syringe. When the volume of gas in the syringe exceeded 60 ml the gas was released by inverting the syringe, opening the clamp on the silicone tube and returning the piston to the volume recorded at time zero. Gas production was recorded at the same time intervals as for the PTT.

At the end of the 144 hour incubation volatile fatty acid (VFA) concentrations and DM loss were determined (as described in sections 3.10.1 and 3.12.1, respectively) for both techniques.

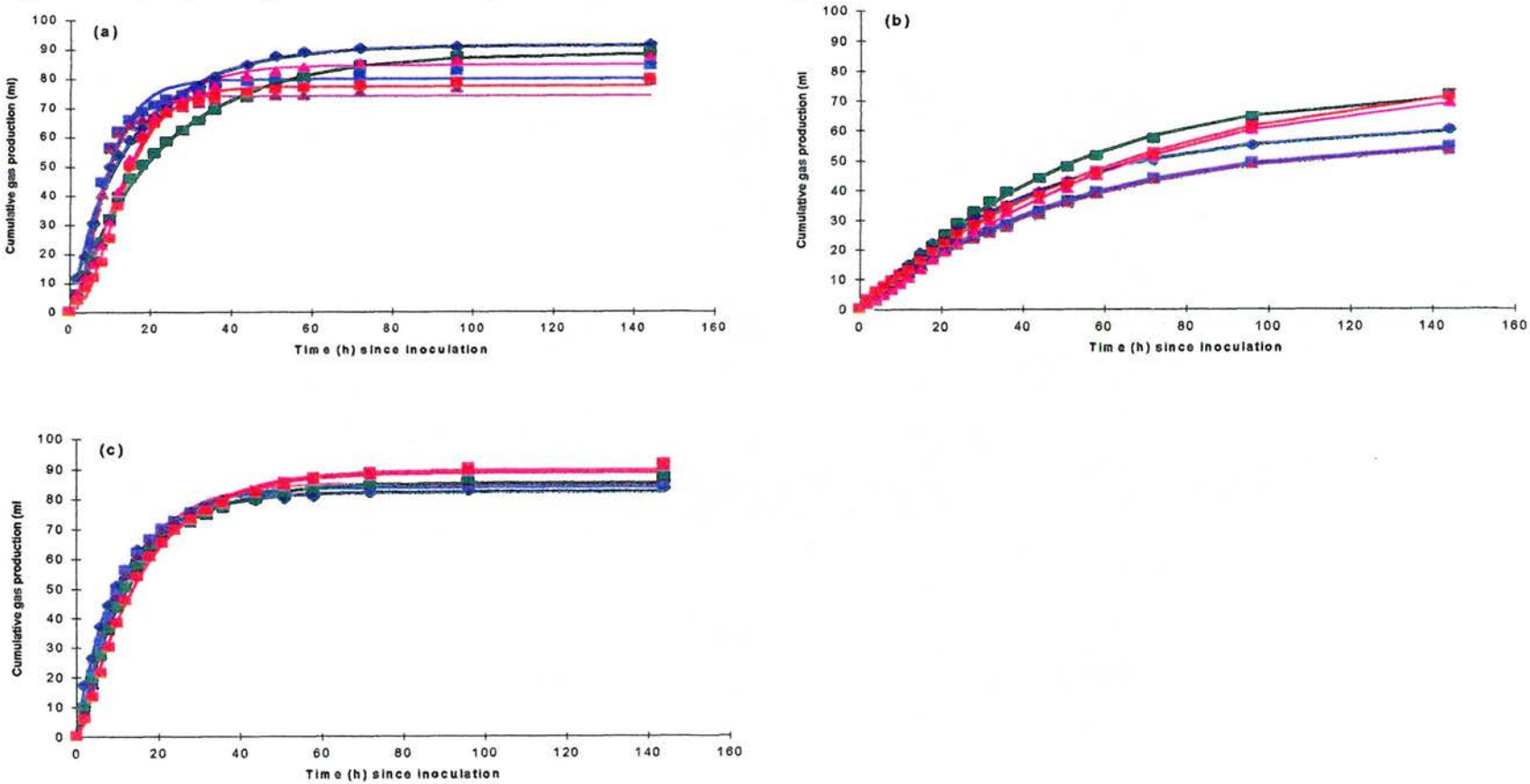
The experiment was a factorial design consisting of 2 different techniques, 3 substrates, 3 M:RF ratios and 3 replicate bottles (2 x 3 x 3 x 3). MLP (Ross, 1987) was used to fit curves to experimentally derived gas accumulation profiles using the model of France *et al.* (1993) (as described in section 3.11). The gas production profiles were then analysed using the parallel curve analysis function of MLP (Ross, 1987). Modelled gas production parameters, DM loss and VFA data were analysed using the analysis of variance function of GENSTAT 5 (Lawes Agricultural Trust, 1993).

7.3 Results

7.3.1 Gas production

The gas production profiles obtained for naked oats, oatfeed and ryegrass using the different techniques and various M:RF ratios are shown in Figure 7.1. Parallel curve analysis (Appendix 7.1) showed that both the rate of gas production and the total cumulative gas production differed significantly between techniques ($p<0.01$).

Figure 7.1 Cumulative gas production profiles for (**a**) naked oats, (**b**) oatfeed, and (**c**) ryegrass during incubation with a medium : rumen fluid ratio of 2:1 (-◆-), 6:1 (-■-) or 9:1 (-▲-) in the Menke technique and incubation with a medium : rumen fluid ratio of 2:1 (-■-), 6:1 (-▲-) and 9:1 (-■-) during incubation in the pressure transducer technique.



Each value represents the mean of three bottles, whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure. Fermentations were conducted in 70 ml serum bottles or 100 ml syringes with 40 ml of medium : rumen fluid in ratios of 2:1, 6:1 and 9:1 as described in section 7.2.

Cumulative gas production was generally highest with the PTT ($p<0.01$); with the exception of naked oats incubated with the 2:1 M:RF ratio.

The fitted parameters and derived quantities for the gas production profiles are shown in Tables 7.2 and 7.3. The rates of gas production, b and c, tended to be greatest during incubation of naked oats, with values ranging across all feeds from 0.0135 h^{-1} (during incubation of oatfeed in the PTT with a M:RF ratio of 9:1) to 0.5270 h^{-1} (during incubation of naked oats in the MT with a M:RF ratio of 9:1) and from -1.7510 $h^{-0.5}$ (during incubation of naked oats in the MT with a M:RF ratio of 9:1) to 0.2050 $h^{-0.5}$ (during incubation of naked oats in the MT with a M:RF ratio of 2:1) for b and c, respectively. The mean values for b and c were 0.0960 ± 0.03029 h^{-1} and -0.1610 ± 0.11119 $h^{-0.5}$ respectively. (b and c were combined to give the fractional rate of degradation, μ, at 12, 24 and 48 h incubation for naked oats, oatfeed and ryegrass (Table 7.4); the values in this table are discussed later in this section). The asymptote of gas production, A, also varied with the different substrates, M:RF ratios and technique used. Values for A ranged from 57.1 ml (during incubation of oatfeed with a 9:1 M:RF ratio in the MT) to 91.9 ml (during incubation of naked oats with a 2:1 M:RF ratio in the MT), with a mean value of 78.81 ± 2.476 ml. The lag time, L_T, ranged from 0.13 h (during incubation of oatfeed with a 9:1 M:RF ratio in the PTT) to 3.18 h (during incubation of naked oats with a 9:1 M:RF ratio in the PTT) with a mean value of 1.15 ± 0.188 h. The time taken to produce 50 and 95 % of the total gas production, t_{50} and t_{95} respectively, ranged from 7.0 h (during incubation of ryegrass with a 2:1 M:RF ratio in the MT) to 47.1 h (during incubation of oatfeed with a 9:1 M:RF ratio in the PTT) and from 16.3 h (during incubation of naked oats with a 9:1 M:RF ratio in the MT) to 212.4 h (during incubation of oatfeed with a 9:1 M:RF ratio in the PTT) for t_{50} and t_{95} respectively. With mean values of 19.26 ± 3.303 h and 78.23 ± 13.950 h, for t_{50} and t_{95} respectively.

Although the gas production profiles produced by the two techniques differed significantly, when ranked according to total gas production, the order of ranking for each technique was similar. However, there were three exceptions (1) naked oats in

Table 7.2 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A)and B for naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer (PTT) or the Menke technique (MT).

Gas production parameter	Technique	M:RF	Substrate NO	OF	RG	s.e.d	T	M	S	TxM	TxS	MxS	TxMxS
b (h^{-1})	PTT	2:1	0.0345	0.0236	0.0585								
		6:1	0.0962	0.0183	0.0713								
		9:1	0.2129	0.0135	0.0699								
	MT	2:1	0.0314	0.0210	0.0690								
		6:1	0.2799	0.0183	0.0776								
		9:1	0.5270	0.0191	0.0857	0.0195	***	***	***	***	***	***	***
c ($h^{-0.5}$)	PTT	2:1	0.074	-0.020	0.080								
		6:1	-0.167	-0.023	-0.035								
		9:1	-0.759	0.009	-0.026								
	MT	2:1	0.205	0.023	0.106								
		6:1	-0.711	0.013	0.074								
		9:1	-1.751	0.004	0.006	0.0948	***	***	***	***	***	***	***
A (ml)	PTT	2:1	88.53	74.10	85.28								
		6:1	85.71	75.91	88.20								
		9:1	83.03	77.07	89.20								
	MT	2:1	91.95	62.33	81.97								
		6:1	78.67	58.32	83.57								
		9:1	72.70	57.09	85.00	3.278	***	NS	***	*	***	***	NS
B	PTT	2:1	100.7	74.2	96.7								
		6:1	79.4	75.4	90.9								
		9:1	37.3	83.4	94.3								
	MT	2:1	117.4	65.8	88.0								
		6:1	47.9	60.6	97.1								
		9:1	16.7	58.8	94.5	8.13	**	***	***	NS	NS	***	**

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 7.1. The s.e.d. is the standard error of the difference for the TxMxS interaction where T = technique, M = medium : rumen fluid ratio and S is the substrate. For each gas production parameter there were 33 degrees of freedom. Significant differences between treatments are shown (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The analysis of variance tables are shown in Appendix 7.2.1 - 7.2.4.

Table 7.3 The gas production parameters; lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer (PTT) or the Menke technique (MT).

Gas production parameter	Technique	M:RF	Substrate NO	OF	RG	s.e.d	T	M	S	TxM	TxS	MxS	TxMxS
L_T (h)	PTT	2:1	1.30	0.37	0.87								
		6:1	1.71	0.20	0.88								
		9:1	3.18	0.13	1.20								
	MT	2:1	1.06	1.33	0.31								
		6:1	1.60	0.84	0.98								
		9:1	2.75	0.95	1.12	0.2914	NS	***	***	NS	***	***	NS
t_{50} (h)	PTT	2:1	15.41	28.64	9.76								
		6:1	11.58	46.45	11.82								
		9:1	12.43	47.07	11.99								
	MT	2:1	9.65	29.91	6.97								
		6:1	7.76	35.84	8.14								
		9:1	7.76	36.46	9.09	1.610	***	***	***	*	*	***	***
t_{95} (h)	PTT	2:1	72.3	123.3	44.3								
		6:1	45.1	147.1	45.7								
		9:1	30.3	212.4	46.2								
	MT	2:1	55.1	134.6	35.3								
		6:1	20.5	157.7	34.9								
		9:1	16.3	151.5	35.5	8.44	***	NS	***	**	NS	***	***

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 7.1. The s.e.d. is the standard error of the difference for the TxMxS interaction where T = technique, M = medium : rumen fluid ratio and S is the substrate. For each gas production parameter there were 33 degrees of freedom. Significant differences between treatments are shown (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The analysis of variance tables are shown in Appendix 7.2.5 - 7.2.7.

the 2:1 M:RF; the PTT ranking it after ryegrass in the 9:1 ratio, whereas the MT ranked it first, (2) naked oats in the 6:1 M:RF ratio was ranked above ryegrass in the 2:1 M:RF ratio in the PTT, whilst the MT ranked it below ryegrass in the 2:1 M:RF ratio and (3) oatfeed in the 9:1 and 2:1 ratios, which were ranked the opposite way in the MT compared to the PTT (Figure 7.2).

Figure 7.2 Order of ranking, from most to least gas, produced during the fermentation of naked oats (NO), oatfeed (OF) and ryegrass (RG) using medium to rumen fluid ratios (M:RF) of 2:1, 6:1 or 9:1 and either the pressure transducer technique (PTT) or the Menke technique (MT).

	PTT	MT
Most gas	RG 9:1	NO 2:1
↓	NO 2:1	RG 9:1
↓	RG 6:1	RG 6:1
↓	NO 6:1	RG 2:1
↓	RG 2:1	NO 6:1
↓	NO 9:1	NO 9:1
↓	OF 9:1	OF 2:1
↓	OF 6:1	OF 6:1
Least gas	OF 2:1	OF 9:1

The fractional rates of degradation (μ) at 12, 24 and 48 h incubation for naked oats, oatfeed and ryegrass are shown in Table 7.4. For naked oats, μ was significantly higher in the 9:1 medium : rumen fluid ratio compared to the 2:1 ratio, whereas for RG and OF μ was greater or similar in the 2:1 ratio compared to the 9:1 ratio. The fractional rate of degradation at 12 h incubation, μ_{12}, ranged from 0.0148 (during incubation of oatfeed with a 9:1 M:RF ratio in the PTT) to 0.2739 h^{-1} (during incubation of naked oats with a 9:1 M:RF ratio in the MT) with a mean value of 0.0727 ± 0.01525 h^{-1}. At 24 h incubation, the fractional rate of degradation, μ_{24}, ranged from 0.0144 (during incubation of oatfeed with a 9:1 M:RF ratio in the PTT)

Table 7.4 The fractional rate of degradation, μ, at 12, 24 and 48 h since inoculation for naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid (M:RF) ratio of 2:1, 6:1 or 9:1 using either the pressure transducer technique (PTT) or the Menke technique (MT).

Fractional rate of gas production	Technique	M:RF	Substrate										
			NO	OF	RG	s.e.d	T	M	S	TxM	TxS	MxS	TxMxS
μ_{12}	PTT	2:1	0.0453	0.0207	0.0700								
		6:1	0.0721	0.0150	0.0663								
		9:1	0.1032	0.0148	0.0661								
	MT	2:1	0.0609	0.0243	0.0843								
		6:1	0.1771	0.0202	0.0883								
		9:1	0.2739	0.0198	0.0865	0.0067	***	***	***	***	***	***	***
μ_{24}	PTT	2:1	0.0421	0.0216	0.0666								
		6:1	0.0791	0.0160	0.0678								
		9:1	0.1355	0.0144	0.0672								
	MT	2:1	0.0522	0.0233	0.0798								
		6:1	0.2073	0.0196	0.0851								
		9:1	0.3483	0.0196	0.0863	0.0102	***	***	***	***	***	***	***
μ_{48}	PTT	2:1	0.0399	0.0222	0.0643								
		6:1	0.0841	0.0167	0.0688								
		9:1	0.1581	0.0142	0.0680								
	MT	2:1	0.0461	0.0227	0.0767								
		6:1	0.2286	0.0192	0.0829								
		9:1	0.4006	0.0194	0.0861	0.0129	***	***	***	***	***	***	***

The fractional rates of degradation were calculated from the b and c values, obtained from fitting the France *et al.* (1993) model to the curves shown in Figure 7.1, using the equation $\mu = b + c / 2\sqrt{t}$. The s.e.d. is the standard error of the difference for the TxMxS interaction where T = technique, M = medium : rumen fluid ratio and S is the substrate. For each gas production parameter there were 33 degrees of freedom. Significant differences between treatments are shown (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The analysis of variance tables are shown in Appendix 7.3.1 - 7.3.3.

to 0.3483 h^{-1} (during incubation of naked oats with a 9:1 M:RF ratio in the MT) with a mean value of 0.0795 ± 0.01950 h^{-1}. Whilst the fractional rate of degradation after 48 h incubation, μ_{48}, ranged from 0.0142 (during incubation of oatfeed with a 9:1 M:RF ratio in the PTT) to 0.4006 h^{-1} (during incubation of naked oats with a 9:1 M:RF ratio in the MT) with a mean value of 0.0844 ± 0.02260 h^{-1}.

The large fractional rates of degradation at 48 h for naked oats in the 9:1 medium : rumen fluid ratio indicate a more sigmoidal gas production profile than that seen with the 2:1 medium : rumen fluid ratio suggesting that the micro-organisms were not in excess in the 9:1 ratio, when first introduced into the culture medium, and require time to multiply. However once they have multiplied they result in a faster rate of gas production than that seen in the 2:1 ratio.

Although the fractional rates of degradation for naked oats were highest in the 9:1 medium the lag time was generally much longer compared to the 2:1 ratio (Table 7.3), suggesting that in the 2:1 ratio the number of micro-organisms present was not limiting whereas in the 9:1 ratio the micro-organisms are more dilute and hence multiply in number before starting to degrade the substrate quickly. The rate at which the 9:1 micro-organisms degrade the substrate, after the lag time has passed, is much quicker than that seen in the 2:1 ratio; as indicated by the fractional rates (Table 7.4) and the time taken to produce 50 % or 95 % of the total gas pool (t_{50} and t_{95} respectively; Table 7.2). Thus, suggesting that the micro-organisms which are suitable for degrading starch are selected for, whereas in the 2:1 ratio the micro-organisms are more general, and not as specific.

For oatfeed, the lag time (L_T), t_{50} and t_{95} tended to increase with the lower microbial concentrations (6:1 and 9:1) suggesting that more micro-organisms were important. For ryegrass, t_{50} and t_{95} also tended to increase with decreasing microbial concentrations, however this pattern was not followed by lag time. L_T being greatest for the 2:1 ratio.

7.3.2 Dry matter loss

The extent of DM loss for naked oats, oatfeed and ryegrass incubated in the pressure transducer technique or the Menke technique are shown in Table 7.5.

Table 7.5 Dry matter loss (mg g^{-1}) for naked oats (NO), oatfeed (OF) and rye grass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using the pressure transducer technique (PTT) or the Menke technique (MT).

Technique	M:RF	DM loss (mg g^{-1})		
		NO	OF	RG
PTT	2:1	$948.1^{a,d}$	549.8^{b}	$923.8^{a,d}$
	6:1	$910.2^{a,e}$	588.0^{b}	968.8^{d}
	9:1	863.2^{e}	515.7^{f}	$940.9^{a,d}$
MT	2:1	$917.0^{a,d}$	$479.1^{c,f}$	$899.8^{a,e}$
	6:1	$932.3^{a,d}$	$458.8^{c,f}$	$898.2^{a,e}$
	9:1	942.7^{a}	473.9^{f}	$900.5^{a,e}$

Each value is the mean of three replicates. Values not bearing the same superscript differ significantly ($p < 0.05$; sed = 25.68). The analysis of variance table is shown in Appendix 7.4.

DM loss varied with technique and feed ($p<0.001$); the PTT generally produced higher values of DM loss than the MT, whilst naked oats and ryegrass were more degradable than oatfeed. There was a significant interaction between technique and feed ($p < 0.001$); DM loss being higher for oatfeed and ryegrass when incubated in the PTT, whilst naked oats was degraded to the same extent by the PTT and MT. There was also a significant interaction between technique and medium : rumen fluid ratio ($p < 0.05$); there was no significant difference in the mean DM loss when incubated in the MT, however degradation in the PTT was significantly higher with the 6:1 ratio compared to the 9:1 ratio (Appendix 7.4).

7.3.3 Volatile fatty acid (VFA) production

Total VFA production ranged from 24.97 mmol l^{-1} (during incubation of oatfeed with a 6:1 M:RF ratio in the MT) to 57.16 mmol l^{-1} (during incubation of ryegrass with the

9:1 M:RF ratio in the PTT), with a mean value of 42.04 ± 2.228 mmol l^{-1} (Table 7.6). Total VFA production was significantly different between techniques with the PTT generally producing more VFA than the MT ($p < 0.001$). There were also significant differences in total VFA production between the feedstuffs; naked oats and ryegrass produced more VFA than oatfeed ($p < 0.001$) (which may be explained by their higher dry matter degradation, i.e. more substrate was degraded resulting in the production of more VFA). Medium : rumen fluid ratio also had a significant effect ($p < 0.05$) and interactions were seen between technique x feed , technique x M:RF, and feed x M:RF (Appendix 7.5.1).

Table 7.6 Total volatile fatty acid (VFA) production (mmol l^{-1}) from naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer technique (PTT) or the Menke technique (MT).

Technique	M:RF	Total VFA (mmol l^{-1})		
		NO	OF	RG
PTT	2:1	$51.10^{a,e}$	41.70^{c}	$50.83^{a,e}$
	6:1	$43.58^{b,c}$	30.64^{d}	$48.34^{a,b}$
	9:1	$45.25^{a,b,c}$	31.21^{d}	57.16^{e}
MT	2:1	$44.18^{b,c}$	28.40^{d}	$41.96^{b,c}$
	6:1	$43.69^{b,c}$	24.97^{d}	$46.68^{a,b,c}$
	9:1	$49.31^{a,b}$	27.61^{d}	$50.06^{a,b}$

Each value is the mean of three replicates. Values not bearing the same superscripts differ significantly ($p < 0.05$; sed = 3.234). The analysis of variance table is shown in Appendix 7.5.1.

The molar percentage of acetate produced varied with the different feedstuffs ($p < 0.001$), techniques ($p < 0.001$) and M:RF ratios ($p < 0.01$) (Table 7.7), ranging from 54.71 % (after incubation of naked oats with the 9:1 M:RF ratio in the PTT) to 77.96 % (after incubation of oatfeed with the 6:1 M:RF ratio in the PTT), with a mean value of 66.70 ± 1.995 % (Table 7.7). Incubation of oatfeed produced the highest molar percentage of acetate, with similar percentages produced by both techniques

and the various M:RF ratios. Incubation of ryegrass produced the next greatest molar percentage of acetate. Incubation in the PTT generally produced higher percentages of acetate than during incubation with the MT, with the exception of incubation with the 9:1 M:RF where the molar percentage of acetate production was greater in the MT. Naked oats had the lowest percentage of acetate at the end of the incubation, again incubation in the PTT generally produced greater percentages of acetate than incubation in the MT, with the exception of the 9:1 M:RF ratio.

Table 7.7 Acetate production (molar %) from naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer technique (PTT) or the Menke technique (MT).

Technique	M:RF	Acetate production (molar %)		
		NO	OF	RG
PTT	2:1	62.07^{a}	75.30^{c}	67.29^{d}
	6:1	58.48^{b}	77.96^{g}	65.95^{h}
	9:1	54.71^{f}	$77.46^{c,g}$	$64.83^{e,h}$
MT	2:1	59.00^{b}	76.50^{c}	$64.86^{e,h}$
	6:1	55.71^{f}	$77.35^{c,g}$	64.32^{e}
	9:1	54.84^{f}	77.89^{g}	66.08^{h}

Each value is the mean of three replicates. Values not bearing the same superscript differ significantly ($p < 0.05$; sed = 0.5839). The analysis of variance table is shown in Appendix 7.5.2.

The molar percentage of propionate produced was also affected by feed, technique and M:RF ratio (Table 7.8; Appendix 7.5.3). The molar percentage of propionate produced ranged from 14.84 % (after incubation of oatfeed with a 2:1 M:RF ratio in the PTT) to 36.82 % (after incubation of naked oats with the 9:1 M:RF ratio in the MT), with a mean value of 22.01 ± 1.733 %. The molar percentage of propionate produced during incubation of oatfeed was similar for both techniques and all M:RF ratios, however differences were seen with naked oats and ryegrass. Incubation with the 2:1 M:RF ratio resulted in higher molar percentages of propionate for both naked oats and ryegrass when the MT was used compared to the PTT. Within techniques,

ryegrass and naked oats resulted in the production of similar molar percentages of propionate, whilst oatfeed produced the lowest molar percentage of propionate during incubation with the 2:1 M:RF. Incubation with the 6:1 M:RF ratio produced similar results to the 2:1 M:RF ratio with incubation of naked oats and ryegrass in the MT producing higher molar percentages of propionate than during incubation in the PTT. The highest molar percentage of propionate within techniques during incubation with the 6:1 M:RF was produced by naked oats, followed by ryegrass with least produced during incubation of oatfeed. A similar pattern was observed during incubation with the 9:1 medium, the highest molar percentage of propionate being produced during incubation of naked oats, followed by ryegrass with least produced during incubation of oatfeed. However, there was no significant difference between techniques with the 9:1 M:RF ratio.

Table 7.8 Propionate production (molar %) from naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer technique (PTT) or the Menke technique (MT).

Technique	M:RF	Propionate production (molar %)		
		NO	OF	RG
PTT	2:1	18.36^{a}	14.84^{c}	$19.37^{a,d}$
	6:1	30.81^{f}	15.10^{c}	$21.19^{b,e,h}$
	9:1	36.13^{j}	15.51^{c}	$22.08^{e,i}$
MT	2:1	$20.25^{b,d}$	15.34^{c}	$21.11^{b,e}$
	6:1	33.21^{g}	15.42^{c}	22.75^{i}
	9:1	36.82^{j}	15.44^{c}	$22.37^{h,i}$

Each value is the mean of three replicates. Values not bearing the same superscript differ significantly ($p < 0.05$; sed = 0.5922). The analysis of variance table is shown in Appendix 7.5.3.

The molar percentage of butyrate produced ranged from 5.27 % (after incubation of oatfeed with the 6:1 M:RF ratio in the MT) to 16.32 % (after incubation of naked oats with the 2:1 M:RF ratio in the MT), with a mean value of 8.46 ± 0.768 % (Table 7.9). During incubation with the 2:1 M:RF ratio there was no difference in the molar

percentage of butyrate produced between techniques for both naked oats and ryegrass, whilst for oatfeed a greater percentage of butyrate was produced during incubation with the PTT compared to the MT ($p < 0.05$). There was also a significant difference in the percentage of butyrate produced by the feedstuffs with naked oats producing the highest molar percentage of butyrate, followed by ryegrass, with least produced by oatfeed ($p < 0.05$). Incubation with the 6:1 and 9:1 M:RF ratios produced similar results, with the highest molar percentage of butyrate produced by ryegrass, followed by naked oats, with least produced by oatfeed. The molar percentage of butyrate production was unaffected by technique for both naked oats and oatfeed, whilst ryegrass produced a significantly higher molar percentage of butyrate during incubation with the PTT compared to the MT ($p < 0.05$).

Table 7.9 Butyrate production (molar %) from naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer technique (PTT) or the Menke technique (MT).

Technique	M:RF	Butyrate production (molar %)		
		NO	OF	RG
PTT	2:1	15.88^{a}	7.37^{b}	$9.78^{d,e}$
	6:1	$8.41^{f,g}$	5.45^{c}	9.35^{d}
	9:1	6.59^{h}	5.48^{c}	$9.50^{d,g}$
MT	2:1	16.32^{a}	5.78^{c}	10.32^{e}
	6:1	8.06^{f}	5.27^{c}	9.04^{g}
	9:1	$5.99^{c,h}$	5.31^{c}	8.35^{f}

Each value is the mean of three replicates. Values not bearing the same superscript differ significantly ($p < 0.05$; sed = 0.3144). The analysis of variance table is shown in Appendix 7.5.4.

Valerate production, as a molar percentage of the total VFA, also varied with the different techniques, feedstuffs and M:RF ratios (Table 7.10). The molar percentage valerate ranged from 1.37 % (after incubation of oatfeed with the 9:1 M:RF ratio in the MT) to 4.42 % (after incubation of naked oats with the 2:1 M:RF ratio in the MT), with a mean value of 2.84 ± 0.213 %. During incubation with the 2:1 M:RF

ratio, naked oats and ryegrass produced similar molar percentages of valerate, whilst oatfeed resulted in a lower percentage of valerate ($p < 0.05$). There was no significant difference in the molar percentage of valerate produced between techniques for both oatfeed and ryegrass, whilst incubation of naked oats in the MT resulted in a higher molar percentage of valerate than during incubation with the PTT ($p < 0.05$). Incubation with the 6:1 M:RF ratio, resulted in the highest molar percentage of valerate for ryegrass, followed by naked oats, with the lowest molar percentage for oatfeed. There was no difference in the molar percentage of valerate produced during incubation of ryegrass between techniques, however incubation of both naked oats and oatfeed in the MT produced significantly higher percentages of valerate than during incubation in the PTT ($p < 0.05$). With the 9:1 M:RF ratio ryegrass resulted in the highest molar percentage of valerate, followed by naked oats, with least produced by oatfeed. There was no difference in the molar percentage of valerate produced between techniques for both naked oats and oatfeed, however incubation of ryegrass in the PTT produced a significantly higher molar percentage than with the MT ($p < 0.05$).

Table 7.10 Valerate production (molar %) from naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer technique (PTT) or the Menke technique (MT).

Technique	M:RF	Valerate production (molar %)		
		NO	OF	RG
PTT	2:1	3.687^{a}	2.485^{c}	3.560^{a}
	6:1	2.520^{c}	1.463^{e}	3.523^{a}
	9:1	2.570^{c}	1.563^{e}	3.577^{a}
MT	2:1	4.423^{b}	2.377^{c}	3.693^{a}
	6:1	3.017^{d}	1.957^{f}	3.865^{a}
	9:1	2.340^{c}	1.370^{e}	3.180^{d}

Each value is the mean of three replicates. Values not bearing the same superscript differ significantly ($p < 0.05$; sed = 0.1786). The analysis of variance table is shown in Appendix 7.5.5.

7.4 Discussion

Cumulative gas production profiles for naked oats, oatfeed and ryegrass varied significantly depending on both the technique used and the medium : rumen fluid ratio. Differences between the techniques may possibly be explained by the nature of recording gas production. In the MT, a certain pressure is required to raise the plunger in the syringe before a volume of gas is recorded, whereas in the PTT there is a fixed head-space area for the gas to occupy so all the gas produced should be measured. The pressure exerted by the plunger in the Menke technique may have the same effect as that induced by longer time intervals between readings in the PTT (section 4.2); where less gas was produced when the bottles were read every 6 h compared to when the bottles were read every 2 or 4 h. However, unlike the results seen when gas production was recorded at different intervals in the PTT, where there was no effect on DM loss, DM loss was significantly lower in the MT compared to the PTT for both oatfeed and ryegrass ($p < 0.001$). This suggests that the amount of substrate degraded, as opposed to the pressure exerted by the syringe plunger, may be the reason for the higher gas production. Although gas production in the PTT was generally higher than the MT, there was the exception of naked oats incubated in the 2:1 M:RF ratio. In addition, the difference in the volume of gas (between the PTT and MT) was not constant suggesting other factors were influencing the gas production profiles. The difference in the gas:liquid surface area between the bottles and syringes will affect the rate at which gas is released from the liquid phase into the head-space (section 2.2.7). The bottles of the PTT have a larger surface area than the syringes in the MT, but again the results obtained were not consistent between feedstuffs nor between medium: rumen fluid ratios.

The effect of medium : rumen fluid ratio was not consistent between feedstuffs. The fractional rate of degradation, μ, was expected to be higher in the 2:1 ratio due to the increased number of micro-organisms per unit of substrate (Pell & Schofield, 1993). Although this was generally observed, there was the exception of naked oats in both the MT and the PTT. A possible explanation for this may be that the VFA profile produced during the incubation of naked oats varied depending upon the M:RF ratio,

with the higher concentration of rumen micro-organisms resulting in increased propionate production hence gas production was slower than in the more dilute culture medium where acetate and butyrate production may have been favoured resulting in higher volumes of gas production (section 2.2.8.1). However, at the end of the incubation propionate production was significantly lower and butyrate production significantly higher in the 2:1 ratio compared to the 9:1 ratio suggesting that the VFA profile was not responsible for the slower rate. Another possible explanation may be that the buffer became exhausted; the large quantity of rumen fluid compared to medium, when rapidly degradable feeds are incubated, may result in exhaustion of the buffer. The large number of micro-organisms which are present in the inoculum will multiply rapidly due to the readily available substrate and may therefore quickly become limited by some other nutrient such as provided by the trace element solution, whereas when the medium is in excess the only limiting factor will be the substrate.

With oatfeed, which is a slowly degradable substrate, the greater concentration of micro-organisms (2:1) resulted in a faster rate of degradation than the lower concentration of micro-organisms (9:1). According to Van Soest (1982) substrate weight should not exceed 1 % of the *in vitro* medium in order to avoid accumulation of end products to levels that will inhibit the fermentation. This specification was met in all M:RF ratios in terms of total culture medium (0.75 %; 300 mg substrate per 40 ml medium + rumen fluid) however in terms of medium alone the 2:1 ratio was slightly higher (1.11 %; 300 mg substrate per 27 ml medium) than the specification although this is not expected to have had a significant effect on gas production. The substrate was also within the limits specified by Cone *et al.* (1996), who found that saturation of the medium occurred (due to exhaustion of the buffer, resulting in a decrease in pH to below 6.5) when more than 0.5 g of a readily fermentable substrate (such as a corn cob mix) was incubated in 60 ml of 2:1 M:RF. This is also in agreement with the findings of Beuvink & Spoelstra (1992), who showed that the system became saturated by the addition of more than 4.5 mmol acetic acid in 60 ml

buffered rumen fluid. Thus, in the experiment reported here it is unlikely that saturation of the buffer occurred.

In general the increased medium concentration : rumen fluid (6:1 or 9:1) caused an increase in lag time (L_T) and the time taken to produce 50 % (t_{50}) or 95 % (t_{95}) of the total gas production (h). This was expected as the higher concentration of micro-organisms in the 2:1 would be expected to increase the instantaneous rate of substrate degradation, hence increasing the rate of gas production and decreasing L_T, t_{50}, and t_{95}. However, this was not seen with naked oats where a decrease in the concentration of micro-organisms, from 2:1 to 6:1 or 9:1, caused a decrease in t_{50} and t_{95}, although L_T was greater. Increased microbial lag times may occur if anaerobic conditions are not present at the start of the incubation (Grant & Mertens, 1992), however the resazurin indicated that this was not the case. Hence the rate of gas production at times greater than L_T, was higher for the lower concentrations of micro-organisms. One possible explanation for this finding may be that if the micro-organisms are limiting when inoculated, the micro-organisms specific for that substrate's components multiply, hence producing an increased rate of gas production to that seen where the microbial population is less specific, for example the 2:1 M:RF, where micro-organisms are in excess making multiplication of the specific micro-organisms (those able to degrade the substrate at the fastest rate) more difficult. The reason this may occur with the naked oats but not oatfeed or ryegrass could be due to the nature of the microbial inoculum. The microbial inoculum was obtained from steers fed perennial ryegrass (*Lolium perrene*) hay *ad libitum* hence the micro-organisms present were adapted to a forage based diet and could degrade the ryegrass (and to some extent the oatfeed) immediately. However the naked oats contain a high level of starch (60 %) and the number of micro-organisms, able to degrade starch, present in the rumen digesta of a forage fed animal will be low (Dr. D.R. Davies, IGER, Aberystwyth - personal communication). Once the starch degrading micro-organisms are established, however, they do not take long to multiply, for example, *Streptococcus bovis* can achieve a doubling time of 14 min (Russell & Hespell, 1981). Cone *et al.* (1996) have also demonstrated the importance of the donor

animal's diet to the fermentation of different feedstuffs; the rate of gas production for a starch based feed being greater when the inoculum was obtained from a starch fed animal as opposed to an animal on a roughage diet. From these results they recommended that if gas production techniques are to be used as feed evaluation tools substrates should be inoculated in rumen fluid collected from an animal on a similar diet. Taking this one step further several authors recommend that a sample of the feed should be incubated in the rumen fluid, to prime the rumen fluid, before using the rumen fluid in a gas production experiment (Harris, 1996; Luchini *et al.*, 1996; Harris & Beever, 1997; Lane & Boying, 1997).

The effect of varying the concentration of rumen fluid for gas production studies has been investigated by both Pell & Schofield (1993) and Sileshi (1994), with conflicting results. Pell & Schofield (1993) found that as the concentration of rumen fluid fell below 20 %, the rate of gas production decreased. However, Sileshi (1994) using a concentration of 5, 10 or 15% rumen fluid, found that there were significant differences in total gas production ($P < 0.05$) between the different levels of rumen fluid, but there were no significant differences in the rate of gas production between the different concentrations of rumen inoculum. The different results obtained by these two authors may be due to the technique used to prepare the microbial inoculum. Where Pell & Schofield (1993) only strained their rumen fluid to obtain the inoculum, Sileshi macerated the rumen fluid before straining. Between 75 % (Forsberg & Lam, 1977) and 95 % (Czerkawski, 1986) of the microbial biomass in the rumen is found associated with feed particles, thus, the number of micro-organisms present in the inoculum of Sileshi is likely to be greater per ml of inoculum than that used by Pell & Schofield (1993) (Johnson *et al.*, 1958; Minato *et al.*, 1966; Cheng *et al.*, 1980; Leedle & Hespell, 1980; Senshu *et al.*, 1980; McAllister *et al.*, 1994; Theodorou *et al.*, 1994). In addition, Craig *et al.* (1984) reported that including particle-associated bacteria with strained ruminal fluid inoculum was more effective at simulating ruminal fermentation of fibre from alfalfa hay than incubation with strained ruminal fluid alone. However, Craig *et al.* (1984) obtained the particle associated micro-organisms by repeated washing of the particle

residue from whole rumen contents with buffered nutrient medium rather than blending in a liquidiser.

The longer lag times observed for naked oats incubated in the 9:1 ratio suggest that the number of micro-organisms was limiting. Although this is the normal ratio of medium : rumen fluid used in the PTT, the method of inocula preparation differs from that used in the MT. In this chapter, the rumen fluid was prepared by straining only, whereas in a normal PTT experiment the rumen fluid is macerated to include those micro-organisms found attached to solid particles. Hence, the increased lag time may not have occurred if the inoculum had been prepared in the normal manner for the PTT. Senshu *et al.* (1980) also reported that when care was taken to ensure all micro-organisms from the digesta were present in their inoculum, the VFA quantity and composition were more closely correlated with *in vivo* data.

The only other comparison of gas production techniques which has been reported is that by Rymer *et al.* (1997a), whereby a ring test was organised with the following institutions; Feed Evaluation and Nutritional Sciences, ADAS Drayton; Institute of Grassland and Environmental Research, Aberystwyth (IGER); Natural Resources Institute, (NRI) Kent; University of Reading; Rowett Research Institute and the University of Wageningen; Institute for Animal Science and Health (ID - DLO), Lelystad and Cornell University. The techniques involved were; Menke *et al.*, 1979 (Rowett Reasearch Institute), Cone *et al.* 1996 (ADAS and ID - DLO), Pell and Schofield, 1993 (Cornell), Theodorou *et al.* 1994 (IGER, NRI, University of Reading and University of Wageningen) and Davies *et al.* 1995 (IGER). A standard protocol was followed by all the institutes, and significant differences were observed in the gas production parameters obtained between techniques. However, when three parameters; asymptote of gas production, time taken to produce half of the total gas production and the rate when 50% of the gas has been produced, were compared the feeds used were generally ranked in the same order.

The major disadvantage of both the manual PTT and the MT is the high labour intensity involved with recording gas production at frequent intervals throughout the incubation period. In order to overcome this problem several authors have developed automated systems for recording gas production (Beuvink *et al.*, 1992; Pell & Schofield, 1993; Davies *et al.*, 1995; Cone *et al.*, 1996) which involve the use of pressure transducers, pressure sensitive switches and computer interfaces (chapter 2). Automation of the recording system has greatly increased the potential of gas production systems as a routine laboratory procedure.

Automation can also remove problems incurred by a build up of pressure in the head-space (chapter 4). The bottles can be set to vent at a pre-determined low pressure, avoiding problems of high pressures in the head-space and providing a standard release of gas for all feeds. For example, feeds which ferment rapidly, such as naked oats, will vent more frequently than slowly fermenting feeds, such as straw, but the maximum pressure in the head-space for either substrate will be the same.

Choosing a system for measuring gas production will depend upon several factors, for example, the available resources (financial, space and time), the reason for gas production studies (i.e. the aim of the experimental work) and the number of experiments planned; the manual system may be cheaper in the short term but if several gas runs are planned it may become expensive in terms of labour.

CHAPTER 8 - POTENTIAL APPLICATIONS FOR GAS PRODUCTION TECHNIQUES

The aim of this chapter was to investigate two potential applications for *in vitro* gas production techniques. Firstly, the ability of the manual pressure transducer technique to predict the *in vivo* dry matter digestibility and digestible energy content of equine feedstuffs was investigated (section 8.1). Secondly the potential of the automated pressure evaluation system (APES; Davies *et al.*, 1995) for evaluating the effects of novel feed additives on rumen fermentation *in vitro* was examined (section 8.2).

8.1 Evaluation of an *in vitro* gas production technique for estimating the *in vivo* digestibility and digestible energy content of equine feeds

8.1.1 Introduction

Nutritional evaluation of feedstuffs and routine prediction of *in vivo* digestibility are essential steps in formulating diets for equines. The use of digestion techniques for ruminants (Hershberger *et al.*, 1959; Tilley & Terry, 1963), to investigate forage digestibility by equines has been reported by both Applegate & Hershberger (1969) and Trevor - Jones *et al.* (1991). Although only a small number of samples were investigated, i.e. alfalfa, timothy and orchard grass (Applegate & Hershberger, 1969) or lucerne chaff and oaten chaff (Trevor - Jones *et al.*, 1991), the DM loss *in vitro* was similar to that *in vivo* ($R^2 = 0.84$; Applegate & Hershberger, 1969). These reported results suggest that ruminant techniques can be applied to predict the digestibility of equine feeds. As discussed in section 2.2, *in vitro* techniques in ruminant nutrition have progressed from end point digestibility measurements (e.g. Tilley & Terry, 1963) to the determination of the kinetics of digestion associated with the fermentation of feedstuffs. This has been achieved by using simple gas measurement techniques, such as the pressure transducer method, to determine the rate and extent of gas production during fermentation of the particular feeds in batch culture (Theodorou *et al.*, 1994; section 2.1.4). In these techniques the microbial

inoculum employed is generally prepared from rumen digesta, necessitating the use of surgically modified (fistulated) animals. However, section 5.3 showed that equine faeces may be a suitable alternative to caecal digesta as a microbial inoculum for gas production studies. The objectives of this study were, therefore, to examine the suitability of an *in vitro* batch culture technique with an inoculum prepared from equine faeces to obtain values associated with the kinetics of degradation and to estimate the apparent *in vivo* digestibility and digestible energy contents of equine feeds.

8.1.2 Materials and Methods

Sixteen feedstuffs; consisting of three samples of lucerne (L1, L2, L3), oat straw (OS), wheat straw (WS), grass haylage (GH), soyabean meal (SBM), barley grain (BG), two soyabean meal / barley grain mixes [33:66 soya / barley grain (SB1) and 66:33 soya / barley grain (SB2)], naked oats (NO), bruised husked oats (BO), rolled naked oats (RNO), an alfalfa / maize mix (AM), soya husks (SH) and a naked oat / oatfeed mix (OF) were ground to pass through a 1 mm dry mesh screen. The chemical composition of these feedstuffs is shown in Appendix 8.1.1. The DM of the feeds ranged from 681 g kg^{-1} (GH) to 969 g kg^{-1} (SB2), with a mean value for all feeds of 873 ± 62.7 g kg^{-1}; acid detergent fibre (ADF) was lowest in the RNO (39 g kg^{-1} DM) and highest in the OS (509 g kg^{-1} DM), with a mean for all feeds of 239 ± 168.9 g kg^{-1} DM; neutral detergent fibre (NDF) ranged from 78 g kg^{-1} DM (RNO) to 762 g kg^{-1} DM (OS), with a mean value for all feeds of 364 ± 247.4 g kg^{-1} DM; crude protein (CP) content was lowest in the OS (37 g kg^{-1} DM) and highest in the SBM (519 g kg^{-1} DM), with a mean value for all feeds of 227 ± 191.3 g kg^{-1} DM; organic matter (OM) content of the feeds ranged from 884 g kg^{-1} DM for L3 to 983 g kg^{-1} DM for NO and RNO, with a mean value for all feeds of 946 ± 30.9 g kg^{-1} DM. The starch content of the feedstuffs ranged from 0 g kg^{-1} DM for L1, L2, L3, OS, WS, GH, SBM and SH to 630 g kg^{-1} DM for RNO, with a mean value across all feeds of 201 ± 61.5 g kg^{-1} DM.

Apparent DM digestibility *in vivo* (DMIV) of the feedstuffs, determined previously (D. Cuddeford & A. Pearson - personal communication), ranged from 443 g kg^{-1} for OS & WS to 920 g kg^{-1} for NO. DMIV values (g kg^{-1}) for the remaining feeds were 610, 650, 752, 541, 700, 675, 670, 680, 780, 880, 644, 741, and 494 for L1, L2, L3, GH, SBM, BG, SB1, SB2, BO, RNO, AM, SH, and OF respectively. The digestible energy content of the feeds, ranged from 7.7 MJ kg^{-1} DM for OS to 17.7 MJ kg^{-1} DM for RNO. DE values (MJ kg^{-1} DM) for the remaining feeds were 9.4, 10.7, 12.2, 10.8, 13.7, 12.4, 12.5, 12.7, 14.8, 17.5, 12.1, 13.7, 8.6 and 9.8 for L1, L2, L3, GH, SBM, BG, SB1, SB2, BO, NO, AM, SH, WS and OF respectively.

Fresh faeces were collected within 1 h of voiding from four ponies which had *ad libitum* access to grass hay and water. The microbial inoculum was prepared as described in section 3.4. Feedstuffs (750 mg) were incubated in triplicate and inoculated with 10 ml of freshly prepared microbial inoculum. Bottles were incubated at 39°C until the end of the 140 h fermentation period. Digestion of feedstuffs was quantified relative to control blanks, which contained microbial inoculum, culture medium and reducing agent but no feed sample. Gas production was recorded at 2, 5, 8, 11, 14, 17, 20, 23, 26, 30, 34, 37.5, 44.5, 52, 59, 72, 96.5, 122 and 140 h after inoculation. After the final reading, pH, VFA production and DM loss were determined (as described in sections 3.10, 3.12.1 and 3.10.1, respectively).

Data were analysed using Genstat 5 (Lawes Agricultural Trust, 1993). Simple linear and quadratic equations for DMIV and DE on each individual variable were obtained. The variables were; (1) cumulative gas volumes at 5 (g5), 11 (g11), 23 (g23), 44.5 (g44), 72 (g72), 96.5 (g96) and 140 (g140) h following incubation, (2) fitted and derived gas production parameters [Q, Z, B, A, T, t_{50}, t_{95} and μ at t_{50} (France *et al.*, 1993)], (3) VFA parameters [total VFA (TVFA; mmol l^{-1}), and acetate (Ac), propionate (Pr) and butyrate (Bu) molar percentages] and (4) DM loss (DML). Stepwise multiple linear regression (SMLR) analysis was also carried out to derive prediction equations from subgroups of individual variables with and without the addition of DM loss and from all available parameters. Equations were constrained to

allow a maximum of three variables plus a constant in order to avoid problems of over fitting.

The residual variation for each prediction equation was partitioned into three components using mean square prediction error (MSPE) analysis (Theil, 1966; Bibby & Toutenburg, 1977);

$$MSPE = (O - P)^2 + S_P^2 (1 - m)^2 + S_O^2 (1 - r^2) \qquad \text{(equation 8.1)}$$

where O and P are the respective means of the actual and predicted values, S_O^2 and S_P^2 are their respective variances, m is the slope of the regression line of O on P and r is their correlation coefficient. The three components are therefore due to (1) mean bias (P - O), (2) line bias; the deviation of the slope (m) of the regression of O on P from unity, and (3) the random variation about this regression line. A positive mean bias indicates that the model generally over predicts relative to the actual values whilst a negative bias tends to under predict relative to the actual values. A large line bias indicates underlying inadequacies in the structure of the model, whilst if the slope of the regression of O on P is less than unity the model tends to under predict at low actual values and over predict at high actual values whilst the opposite occurs if the regression of O on P is greater than unity. For each equation the MSPE is presented in terms of the proportional contribution of each of these three components. The square root of the MSPE is the mean prediction error (MPE) and is reported as a proportion of the mean actual DMIV or DE observed *in vivo*.

For consistency of style in the prediction equations μ (h^{-1}), the time dependent fractional rate, at the time when 50% of the total gas has been produced, is referred to as the fractional rate of gas production (FRGP).

8.1.3 Results

8.1.3.1 Gas production

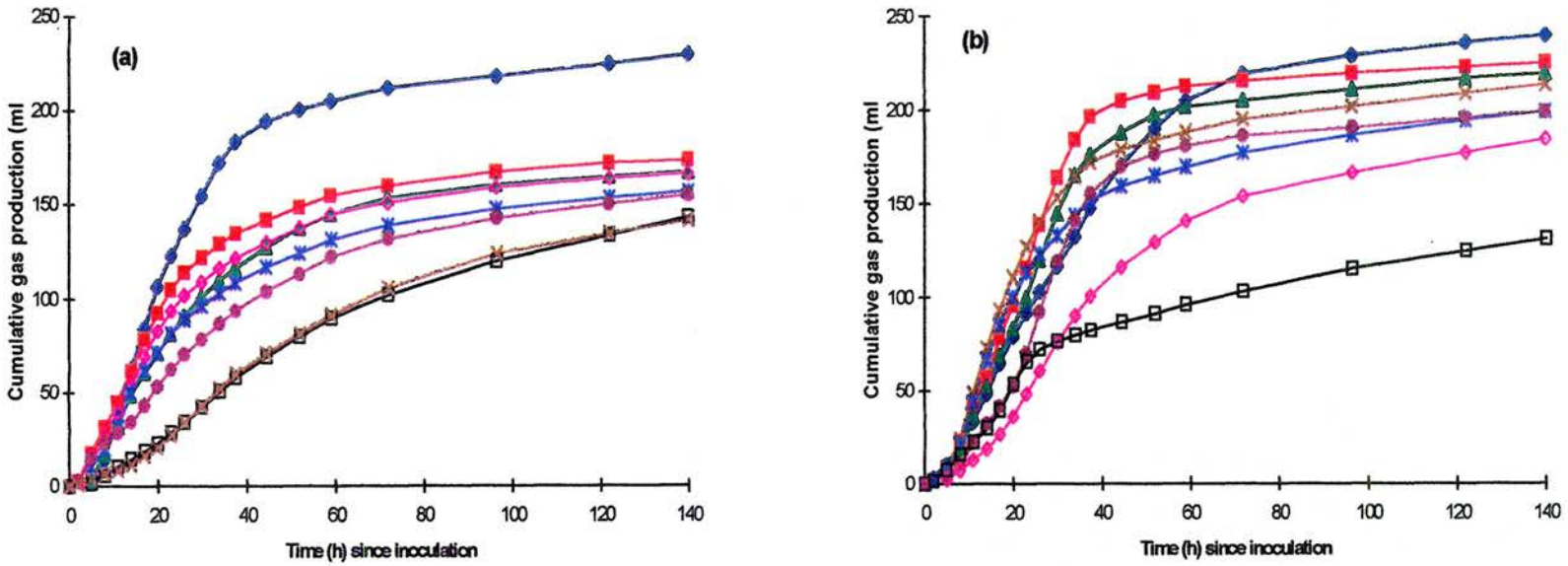
A wide range of gas accumulation profiles between the feedstuffs were observed (Figures 8.1.1a and b). Estimates from fitted curves for parameter values and derived quantities were determined according to France *et al.* (1993) and these are shown in Table 8.1.1.

The lag time, L_T (h), encountered prior to active fermentation varied with the different feeds, ranging from 1.04 h for GH to 7.58 h for BO, with a mean value for all feeds of 4.15 ± 1.71 h. However, the lag time for some feeds may be an overestimate, particularly where starch and/or sugar contents are high resulting in enhanced propionate production without the concomitant production of gas and hence the appearance of an apparently long lag time. The rates of gas production, b and c, ranged from 0.0263 h^{-1} (OF) to 0.1188 h^{-1} (BHO) and -0.6539 $h^{-0.5}$ (BHO) to 0.0629 $h^{-0.5}$ (SB2), respectively. The mean value for b for all feeds was 0.0588 ± 0.0320 h^{-1} whilst that for c was -0.1865 ± 0.2118 $h^{-0.5}$. The fractional rate of gas production, FRGP, ranged from 0.0197 h^{-1} (OS) to 0.0622 h^{-1} (RNO), with a mean value for all feeds of 0.0403 ± 0.0132 h^{-1}. In all but two feeds, > 50 % of the total gas production, GP, was produced within the first 35 h of the incubation and the time taken to produce 50 % of the GP (t_{50}) ranged from 18.55 h (SB1) to 49.69 h (OS), with a mean value for all feeds of 27.18 ± 9.17 h. In the two exceptions, OS and WS, 50 h of incubation was required to produce *ca.* 50 % of the GP. Incubation of OF produced the least gas (127.8 ml) whilst SH produced most gas (242.0 ml) during the 140 h incubation period. The mean total gas production for all feeds was 181.8 ± 31.46 ml (Table 8.1.1).

8.1.3.2 Dry matter loss

DM loss values were least during incubation of the lucerne sample L2 (382 mg g^{-1}), and greatest with the soyabean meal, SBM (966 mg g^{-1}), during the 140 h incubation. Values for apparent DM loss (mg g^{-1}) were as follows for the remaining feeds; GH

Figure 8.1.1 Cumulative gas production for **(a)** three samples of lucerne (L1, -✳-; L2, -◊-; L3, -■-), oat straw (OS, -□-), wheat straw (WS, -X-), grass haylage (GH, -●-), soyabean meal (SBM, -▲-), barley grain (BG, -◆-) and **(b)** two soyabean meal / barley grain mixes (SB1, -X- and SB2 -✳-), soya hulls (SH, -◆-), naked oats (NO, -▲-), bruised oats (BHO, -●-), rolled naked oats (RNO, -■-), alfalfa / maize (AM, -◊-) and a naked oat / oatfeed mix (OF, -□-) during a 140 h incubation with a microbial inoculum prepared from fresh pony faeces.



Fermentations were conducted in 160 ml serum bottles with 89 ml of culture medium and 10 ml of microbial inoculum prepared from freshly voided equine faeces. Each value represents the mean of three bottles whilst the lines indicate the profile as described by France *et al.* (1993). In all cases SE were less than 5 % of the values shown in the figure.

Table 8.1.1. The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for the sixteen feeds incubated with an inoculum obtained from pony faeces.

Feed	Gas production parameters							
	b (h^{-1})	c ($h^{-0.5}$)	A (ml)	B	L_T (h)	t_{50} (h)	t_{95} (h)	FRGP (h^{-1})
L1	0.0325	0.0020	153.7	172.7	3.48	24.52	94.84	0.0327
L2	0.0471	-0.0592	163.0	163.4	1.69	20.53	74.70	0.0405
L3	0.0576	-0.0953	169.2	164.6	1.64	18.61	64.50	0.0466
OS	0.0284	-0.1222	151.3	129.0	4.63	49.69	155.20	0.0197
WS	0.0315	-0.1363	148.6	126.8	4.68	47.28	143.56	0.0215
GH	0.0336	-0.0686	156.0	146.4	1.04	30.00	70.53	0.0273
SBM	0.0367	-0.0020	167.1	198.5	4.81	23.75	86.53	0.0365
BG	0.0856	-0.2852	221.1	179.4	5.04	20.99	58.17	0.0544
SB1	0.0704	-0.1345	204.5	210.5	4.43	18.55	57.66	0.0548
SB2	0.0377	0.0629	192.9	267.0	4.92	19.66	73.90	0.0448
NO	0.1031	-0.4756	214.8	117.8	5.32	23.04	58.50	0.0536
BO	0.1188	-0.6539	194.9	77.2	7.58	26.06	59.80	0.0550
RNO	0.1278	-0.6043	222.2	103.3	5.59	21.13	51.21	0.0622
AM	0.0555	-0.2716	180.3	127.2	5.98	34.96	94.90	0.0326
SH	0.0481	-0.1638	242.0	205.8	2.89	29.75	91.95	0.0331
OF	0.0263	0.0243	127.8	143.0	2.75	26.39	110.38	0.0287

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 8.1.1. Q and Z (the negative logarithms of b and c respectively) were used in regression equations.

483; WS 513; OF 520; L1 549; OS 554; L3 564; BO 604; AM 650; BG 794; SH 809; SB2 902; SB1 917; RNO 944 and NO 945.

8.1.3.3 Volatile fatty acid (VFA) production

Values for the total concentration (mmol l^{-1}) of VFA produced during incubations are shown in Table 8.1.2, together with values for the acetate, propionate and butyrate molar percentages. Production of total VFA was highest after incubation of SH (59.8 mmol l^{-1}) and lowest after incubation of WS (29.9 mmol l^{-1}), with a mean value for all feeds of 43.2 ± 5.54 mmol l^{-1}. The molar percentages varied considerably between feeds. The molar percentages for individual acids ranged from 51.4 (RNO) to 66.3 (L3), 27.6 (SB2) to 41.7 (RNO), and 4.8 (WS) to 12.2 (SBM), for acetate, propionate and butyrate respectively. This gave a mean molar percentage across all feeds of 60.3 ± 2.43 acetate, 32.6 ± 2.21 propionate and 7.1 ± 1.33 butyrate.

8.1.3.4 Prediction Equations

There were several significant correlations between the predictive parameters (Table 8.1.3). For example, cumulative gas volumes greater than g5 were all positively correlated, as might be expected from the summative nature of these measurements. Positive correlations between A and most of the cumulative gas volumes, Z and FRGP were also seen. t_{50} was positively correlated with t_{95} whilst both t_{50} and t_{95} were negatively correlated with most of the cumulative gas volumes and FRGP. Q was negatively correlated with both Z and FRGP, whilst Z was positively correlated with FRGP. Again, high degrees of correlation were expected between these parameters since both Q and Z are fitted parameters which quantify the rate of gas production whilst FRGP is derived from the negative logarithms of Q and Z. Total VFA was positively correlated with cumulative gas volumes greater than g5, B, A and Bu, but not Ac or Pr. Ac and Pr were negatively correlated indicating that variation in fermentation pattern between feeds was mainly expressed by changes in the Ac to Pr ratio. DM loss was positively correlated with cumulative gas volumes greater than g5, A, FRGP, total VFA and Bu, but negatively correlated with Ac.

Table 8.1.2. Volatile fatty acid (VFA) molar percentages and total VFA concentrations (mmol l^{-1}) in culture filtrates collected at the end of 140 h of incubation of sixteen feeds with an inoculum obtained from pony faeces.

Feeds	VFA molar percentages			Total VFA
	Acetate	Propionate	Butyrate	(mmol l^{-1})
L1	64.3 (1.07)	30.4 (0.95)	5.3 (0.17)	36.39 (1.157)
L2	65.2 (0.73)	29.5 (0.72)	5.3 (0.10)	33.66 (0.903)
L3	66.3 (0.51)	28.5 (0.52)	5.2 (0.10)	42.80 (0.385)
OS	60.0 (0.75)	35.0 (0.69)	5.0 (0.07)	33.86 (0.782)
WS	61.9 (0.26)	33.3 (0.26)	4.8 (0.01)	29.94 (0.286)
GH	61.7 (0.20)	33.4 (0.16)	5.0 (0.05)	35.52 (0.049)
SBM	58.4 (0.49)	29.4 (0.51)	12.2 (0.02)	53.33 (0.493)
BG	57.1 (0.67)	33.4 (0.40)	9.5 (0.34)	50.38 (1.759)
SB1	61.1 (1.05)	30.0 (0.92)	8.9 (0.29)	53.87 (0.150)
SB2	62.4 (0.75)	27.6 (0.55)	9.9 (0.25)	56.22 (1.067)
NO	53.7 (0.89)	38.6 (0.65)	7.7 (0.26)	49.55 (1.407)
BO	55.0 (0.26)	35.3 (0.41)	9.7 (0.22)	36.60 (0.365)
RNO	51.4 (0.39)	41.7 (0.76)	6.9 (0.38)	42.54 (0.790)
AM	63.1 (0.27)	30.5 (0.18)	6.4 (0.17)	44.61 (0.214)
SH	60.0 (0.67)	34.9 (0.47)	5.1 (0.22)	59.81 (1.276)
OF	63.1 (0.19)	30.7 (0.17)	6.3 (0.11)	32.22 (0.950)

Each value is the mean of three samples, values in parenthesis represent the standard error.

Tables 8.1.4 and 8.1.5 show the significant linear and quadratic relationships ($p < 0.05$) obtained between the individual parameters and DMIV and DE, respectively. Despite statistical significance, prediction of both DMIV and DE (Figure 8.1.2.a) from DM loss alone was poor indicating the limitations of predicting *in vivo* parameters from end point *in vitro* digestion techniques. g44 was the best single predictor of both DMIV and DE (Figure 8.1.2.b).

Table 8.1.3. Correlation matrix for cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss following incubation of sixteen equine feeds.

g11	0.470																		
g23	0.279	0.958																	
g44	0.006	0.733	0.861																
g72	-0.019	0.653	0.788	0.974															
g96	-0.063	0.594	0.742	0.949	0.993														
g140	-0.110	0.560	0.719	0.932	0.983	0.994													
Q	0.183	-0.334	-0.492	-0.808	-0.750	-0.730	-0.711												
Z	-0.319	-0.017	0.146	0.577	0.550	0.547	0.536	-0.928											
B	0.193	0.533	0.466	0.132	0.163	0.159	0.174	0.453	-0.687										
A	-0.137	0.496	0.652	0.894	0.965	0.979	0.992	-0.687	0.543	0.149									
t50	-0.419	-0.903	-0.893	-0.714	-0.606	-0.542	-0.482	0.403	-0.087	-0.373	-0.390								
t95	-0.320	-0.798	-0.834	-0.852	-0.773	-0.726	-0.670	0.680	-0.426	-0.106	-0.596	0.881							
L_T	-0.835	-0.230	-0.031	0.336	0.326	0.355	0.373	0.573	-0.658	-0.309	0.375	0.124	-0.063						
FRGP	0.015	0.720	0.830	0.925	0.825	0.783	0.753	-0.867	0.630	-0.020	0.689	-0.758	-0.873	0.388					
TVFA	-0.123	0.582	0.652	0.665	0.740	0.756	0.762	-0.209	-0.007	0.645	0.750	-0.466	-0.460	0.190	0.440				
Ac	0.526	-0.077	-0.231	-0.593	-0.574	-0.560	-0.574	0.780	-0.812	0.412	-0.601	0.074	0.342	-0.651	-0.556	-0.239			
Pr	-0.284	-0.133	-0.016	0.358	0.376	0.378	0.403	-0.654	0.778	-0.634	0.461	0.195	-0.111	0.371	0.292	-0.074	-0.838		
Bu	-0.487	0.357	0.443	0.483	0.417	0.388	0.373	-0.336	0.191	0.294	0.325	-0.457	-0.441	0.566	0.526	0.551	-0.434	-0.128	
DML	-0.399	0.499	0.599	0.700	0.706	0.707	0.713	-0.455	0.282	0.341	0.706	-0.398	-0.445	0.483	0.583	0.831	-0.616	0.264	0.67
	g5	g11	g23	g44	g72	g96	g140	Q	Z	B	A	t50	t95	L_T	FRGP	TVFA	Ac	Pr	B

Coefficients > 0.497 are significant ($P < 0.05$). For abbreviations see section 8.1.2.

Table 8.1.4. Linear and quadratic relationships between dry matter digestibility *in vivo* (DMIV) and cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss.

		Linear $y = a + bx$				Quadratic $y = a + bx + cx^2$					
x	a	b	R^2	RSD	Significance	a	b	c	R^2	RSD	Significance
g5											
g11	458.8	6.610	0.328	112	*	334	17.80	-0.201	0.325	112	*
g23	420.1	2.949	0.423	104	**	219	9.11	-0.0397	0.467	99.5	**
g44	283.5	2.743	0.751	68	***	104	5.66	-0.0107	0.752	67.9	***
g72	194.9	2.884	0.708	73.6	***	-256	8.85	-0.0186	0.727	71.3	***
g96	141.5	3.027	0.661	79.4	***	-558	11.56	-0.0249	0.688	76.2	***
g140	102	3.060	0.580	88.3	***	-1270	18.33	-0.0411	0.645	81.2	***
Q	4017	-3533	0.616	84.5	***	-27719	64828	-36790	0.632	82.7	***
Z	305	291.4	0.370	108	**	335	247	16	0.322	112	*
B											
A	89	3.168	0.537	92.7	***	-1008	15.34	-0.0328	0.568	89.6	**
t50	918.6	-9.330	0.379	107	**	794	-1.0	-0.124	0.338	111	*
t95	959.8	-3.504	0.591	87.2	***	1105	-6.75	0.0161	0.574	89	**
L_T											
FRGP	317.4	8634	0.675	77.7	***	111	19925	-138837	0.676	77.6	***
TVFA	327	7.820	0.253	118	*	-1454	91.8	-0.946	0.473	99	**
Ac	1814	-19.07	0.300	114	*	13935	-433	3.51	0.500	96.4	**
Pr						5065	-275	4.23	0.349	110	*
Bu											
DML	361	0.439	0.364	109	**	597	-0.28	0.00051	0.326	112	*

For abbreviations see section 8.1.2.

Table 8.1.5. Linear and quadratic relationships between *in vivo* digestible energy content (DE) and cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss.

		Linear $y = a + bx$				Quadratic $y = a + bx + cx^2$					
x	a	b	R^2	RSD	Significance	a	b	c	R^2	RSD	Significance
g5											
g11	8.73	0.1137	0.203	2.53	*						
g23	7.75	0.0545	0.319	2.34	*	4.34	0.1590	-0.000675	0.326	2.32	*
g44	4.58	0.05532	0.704	1.54	***	4.84	0.0510	0.000016	0.682	1.60	***
g72	2.77	0.0583	0.668	1.63	***	-0.32	0.099	-0.000127	0.647	1.68	***
g96	1.61	0.0617	0.634	1.71	***	-4.9	0.141	-0.000231	0.616	1.75	***
g140	0.70	0.0629	0.567	1.86	***	-17.1	0.261	-0.000532	0.571	1.85	**
Q	83.8	-75.8	0.654	1.67	***	-105	331	-219	0.631	1.72	***
Z	4.07	6.65	0.461	2.08	**	6.5	2.9	1.30	0.421	2.15	*
B											
A	0.32	0.0658	0.537	1.93	***	-14.2	0.227	-0.000434	0.528	1.94	**
t50	16.82	-0.1673	0.264	2.43	*						
t95	18.11	-0.0693	0.530	1.94	***	21.96	-0.155	0.000426	0.517	1.97	**
L_T											
FRGP	5.35	172.0	0.615	1.76	***	5.04	189	-211	0.586	1.82	***
TVFA	5.32	0.1609	0.247	2.46	*	-26.4	1.658	-0.01686	0.393	2.21	*
Ac	42.08	-0.494	0.508	1.99	***	224.8	-6.73	0.0529	0.604	1.78	***
Pr	-0.37	0.387	0.223	2.50	*	95.8	-5.31	0.0833	0.441	2.12	**
Bu											
DML	5.11	0.01033	0.487	2.03	**	10.70	-0.0068	0.0000121	0.463	2.08	**

For abbreviations see section 8.1.2.

Multiple linear regression equations obtained using the individual subgroups of parameters and all available parameters are shown in Tables 8.1.6 and 8.1.7 for DMIV and DE respectively. Using cumulative gas production volumes alone to predict DMIV (equations 8.2 and 8.3) and DE (equation 8.13) resulted in predictive equations with higher R^2 and lower RSD values than any of the linear or quadratic equations shown in Tables 8.1.4 and 8.1.5. Including DM loss with cumulative gas production volumes improved the prediction of DE (equation 8.14), whilst the prediction of DMIV was not improved when DM loss was included (equation 8.4).

Using fitted and derived gas production parameters to predict both DMIV and DE was generally less accurate than using the cumulative gas volumes (equation 8.5 & 8.6 for DMIV and equation 8.15 for DE). Including DM loss as a variable in the equation improved the prediction of both DMIV (equation 8.7 & 8.8) and DE (equations 8.16 & 8.17; Figure 8.1.2.c) compared to their prediction from fitted and derived gas production parameters alone (equations 8.6 and 8.15 for DMIV and DE respectively).

Prediction equations with the highest R^2 and lowest RSD values for both DMIV (equation 8.9) and DE (equation 8.19) were obtained from VFA parameters alone. Incorporating DM loss as a predictor did not improve these equations (equations 8.10 and 8.20, for DMIV and DE respectively).

When all available parameters were made available to the SMLR procedure equations 8.9 and 8.19 proved to be the best predictors of DMIV and DE respectively in terms of R^2 and RSD values. However, additional equations incorporating cumulative gas volumes, fitted and derived gas production parameters, VFA parameters and DM loss are also shown in Tables 8.1.6 and 8.1.7 as examples of alternative prediction relationships.

The mean predicted DMIV and DE and their standard errors for equations 8.2 - 8.24 are shown in Table 8.1.8 together with the MSPE, and the proportion of MSPE

Table 8.1.6. Prediction of dry matter digestibility *in vivo* (DMIV) from cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss.

Parameters	Equation no.	Prediction equation	R^2	RSD
Cumulative gas volumes only	8.2	y = -1039 + 349 log g44	0.771	65.3
	8.3	y = 234.2 + 4.907 g44 - 8.15 g23 + 13.63 g11	0.836	55.3
	8.4	y = -1539 - 1.87 g23 + 0.061 DML + 474 log g44	0.791	62.4
Fitted and derived gas production parameters	8.5	y = 1746 + 331 log FRGP	0.705	74.1
	8.6	y = 155 + 1.50 A + 6209 FRGP	0.720	72.1
	8.7	y = 1191 + 1.142 A + 0.065 DML + 238.3 log FRGP	0.733	70.4
	8.8	y = 1261 + 0.160 DML + 225.4 log FRGP + 159.0 log Z	0.755	67.5
VFA parameters	8.9	y = 15431 - 535.8 Ac + 4.426 Ac^2 + 368.1 log TVFA	0.859	51.2
	8.10	y = 83213 + 0.372 DML + 448 Ac - 26807 log Ac	0.663	79.1
All available parameters		Equations 8.3 and 8.9 were selected.		
	8.11	y = 7106 - 256.6 Ac + 2.11 Ac^2 + 270.9 log g44	0.856	51.8
	8.12	y = -975 + 0.055 DML + 328.1 log g44	0.757	67.2

For abbreviations see section 8.1.2. All equations shown are significant ($p < 0.001$).

Table 8.1.7. Prediction of digestible energy content (DE) from cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss.

Parameters	Equation no.	Prediction equation	R^2	RSD
Cumulative gas volumes	8.13	$y = -2.65 + 0.0941\ g44 - 0.1112\ g23 + 3.32 \log g11$	0.788	1.30
	8.14	$y = -32.91 - 0.0543\ g23 + 0.00429\ DML + 9.57 \log g44$	0.799	1.27
Fitted and derived gas production parameters	8.15	$y = 4.36 + 0.0295\ A + 4.60\ Z - 0.1152\ t50$	0.768	1.36
	8.16	$y = 4.78 + 0.00629\ DML - 53.3 \log Q$	0.803	1.26
	8.17	$y = -0.68 + 0.01087\ DML + 6.82\ Z - 2.297 \log L_T$	0.878	0.99
VFA parameters	8.18	$y = 133 - 29.48 \log Ac$	0.527	1.95
	8.19	$y = 251.4 - 8.57\ Ac + 0.0692\ Ac^2 + 6.55 \log TVFA$	0.865	1.04
	8.20	$y = 1324 + 0.00727\ DML + 6.92\ Ac - 423 \log Ac$	0.761	1.38
All available parameters		Equations 8.16, 8.17 and 8.19 were selected.		
	8.21	$y = 42.8 - 0.1209\ g44 + 19.13 \log g44 - 26.14 \log Ac$	0.856	1.08
	8.22	$y = 222.9 - 493\ FRGP + 21.87 \log FRGP - 29.13 \log Ac$	0.852	1.09
	8.23	$y = 201.7 + 0.0419\ A - 6.4\ Ac + 0.0518\ Ac^2$	0.754	1.40
	8.24	$y = 387 + 0.0478\ A + 4.78\ Pr - 155.1 \log Pr$	0.689	1.58

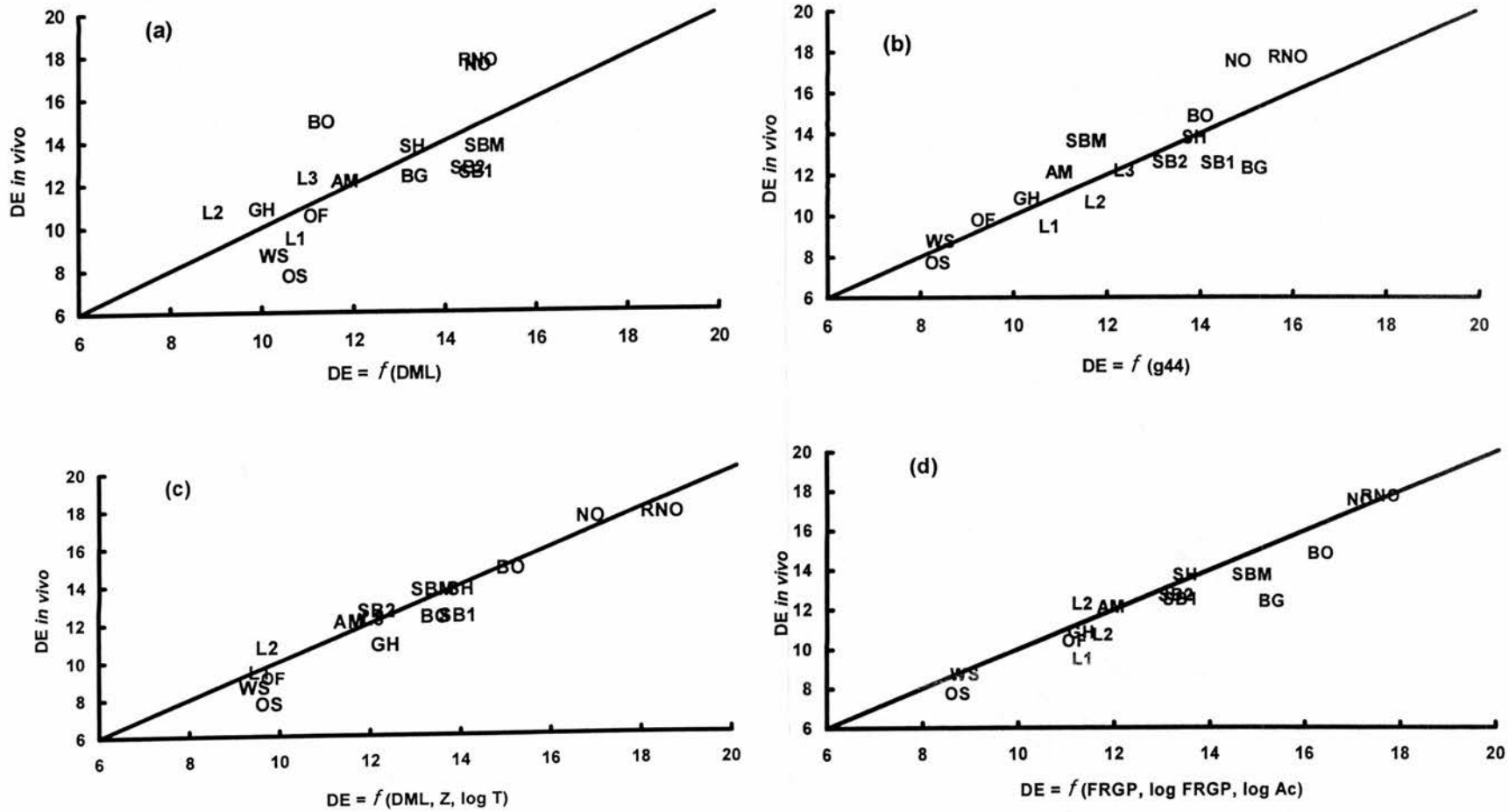
For abbreviations see text. All equations shown are significant ($p < 0.001$).

Table 8.1.8. Prediction precision of equations 8.2 - 8.12 for predicting dry matter digestibility *in vivo* (DMIV) and for equations 8.13 - 8.24 for predicting the digestible energy (DE) content of sixteen equine feedstuffs.

		Predicted				Proportion of MSPE			
Equation no[§]		Mean	s.e.	MSPE[†]	Bias	Line	Random	Bias[‡]	MPE
DMIV	8.2	665	30.2	3596	<0.001	<0.001	0.999	<0.1	0.090
	8.3	665	31.8	2443	<0.001	<0.001	0.999	<0.1	0.074
	8.4	662	31.1	3118	0.002	<0.001	0.998	-2.5	0.084
	8.5	665	29.0	5131	<0.001	<0.001	0.999	<0.1	0.108
	8.6	678	30.1	4512	<0.001	<0.001	0.999	<0.1	0.101
	8.7	665	30.2	3969	<0.001	<0.001	0.999	<0.1	0.095
	8.8	665	30.5	3647	<0.001	<0.001	0.999	<0.1	0.091
	8.9	667	32.1	2099	0.002	<0.001	0.998	2.0	0.069
	8.10	660	29.2	5039	0.005	<0.001	0.995	-5.0	0.107
	8.11	663	32.1	2151	0.002	<0.001	0.998	-2.1	0.070
	8.12	665	30.3	3911	<0.001	<0.001	0.999	<0.1	0.094
DE	8.13	12.26	0.644	1.359	<0.001	<0.001	0.999	<0.01	0.095
	8.14	12.28	0.649	1.289	<0.001	<0.001	0.999	<0.01	0.093
	8.15	12.27	0.639	1.488	<0.001	<0.001	0.999	<0.01	0.099
	8.16	12.28	0.645	1.366	<0.001	<0.001	0.999	<0.01	0.095
	8.17	12.27	0.672	0.783	<0.001	<0.001	0.999	<0.01	0.072
	8.18	12.24	0.529	3.541	<0.001	<0.001	0.999	<0.01	0.153
	8.19	11.91	0.674	0.996	0.129	<0.001	0.870	-0.36	0.081
	8.20	13.31	0.635	2.608	0.413	<0.001	0.586	1.04	0.132
	8.21	12.31	0.666	0.928	0.002	<0.001	0.997	0.04	0.079
	8.22	12.28	0.665	0.953	<0.001	<0.001	0.999	<0.01	0.080
	8.23	12.61	0.629	1.689	0.067	<0.001	0.932	0.26	0.106
	8.24	11.93	0.614	2.113	0.056	<0.001	0.944	-0.24	0.118

[§] For equation details see Tables 8.1.6 and 8.1.7.
[†] $(g\ kg^{-1})^2$ for equations 8.2 - 8.12 and $(MJ\ kg^{-1})^2$ for equations 8.13 - 8.24.
[‡] $g\ kg^{-1}$ for equations 8.2 - 8.12 and $MJ\ kg^{-1}$ for equations 8.13 - 8.24.

Figure 8.1.2 The relationship between digestible energy content (DE) and **(a)** dry matter loss *in vitro*, DML (R^2 = 0.487; RSD = 2.03), **(b)** cumulative gas volume at 44.5 h incubation, g44 (R^2 = 0.704; RSD = 1.54), **(c)** gas production parameters, Z and log T, and DML (equation 8.17; R^2 = 0.878; RSD = 0.99) and **(d)** FRGP, log FRGP and Ac (equation 8.22; R^2 = 0.852; RSD = 1.09).



attributable to mean bias, line bias and random error. The actual mean bias for each equation and the MPE expressed as a proportion of the mean observed DMIV or DE are also shown. Random error accounted for more than 0.99 of the MSPE in all equations except 8.19, 8.20, 8.23 and 8.24. For the majority of DMIV equations the actual predictive bias was negligible; the exceptions were a small positive bias of 2.0 g kg^{-1} for equation 8.9 and a small negative bias of 2.5, 5.0 and 2.1 g kg^{-1} for equations 8.4, 8.10 and 8.11 respectively. For the prediction of DE actual predictive bias was also negligible for the majority of predictive equations with the exceptions of equations 8.20, 8.21 and 8.23, which had positive bias of 1.04, 0.04 and 0.26 MJ kg^{-1} DM, respectively and equations 8.19 and 8.24 which showed negative bias of 0.36 and 0.24 MJ kg^{-1} DM respectively. MPE ranged from 0.069 (equation 8.9) to 0.108 (equation 8.5) for DMIV and from 0.072 (equation 8.17) to 0.153 (equation 8.18) for DE.

8.1.4 Discussion

The cumulative gas production profiles of the feeds reflected their wide diversity, demonstrating the potential of the pressure transducer technique to evaluate digestion kinetics of a diverse array of equine feedstuffs. Equine faeces proved to be a suitable source of inoculum for gas production studies, producing typical gas production profiles for the sixteen feedstuffs under investigation. Macheboeuf and Jestin (1997) have also reported the use of equine faeces as a source of microbial inoculum in gas production studies. Using the Menke and Steingass (1988) technique they concluded that equine faeces and equine caecal fluid were equally good as sources of microbial inoculum for predicting the *in vivo* organic matter digestibility (OMD) of 52 forages in horses. Using faeces to replace rumen fluid as a microbial inoculum was first reported by El Shaer *et al.* (1987) to determine the *in vitro* digestibility of a number of samples of grass, grass silage, lucerne, barley straw, soyabean meal and rolled barley. They used a modified version of the two-stage Tilley and Terry technique (1963) and obtained digestibility results *in vitro* which were closely correlated (R^2 = 0.98) with the *in vivo* digestibilities; the relationship was represented by the equation; *in vivo* digestibility = *in vitro* digestibility x 1.003. However, the technique employed

was modified according to the nature of the substrate to be fermented, for example, a nitrogen source was added to all feedstuffs and the fermentation time for the barley straw was increased from 48 to 72 h. The ability of faeces to replace rumen fluid in Tilley and Terry incubations has also been reported by Omed *et al.* (1989) and Akhter *et al.* (1994). In the latter study, the results were not compared to *in vivo* apparent digestibilities but the authors did find a high correlation ($R^2 = 0.97$) between *in vitro* digestibility determined using sheep rumen fluid and *in vitro* digestibility determined using freshly voided cow faeces. Moreover, the relationship held even after freezing and thawing of the faeces. Using cow faeces in the pressure transducer technique, as an alternative to rumen fluid has been investigated by Harris *et al.* (1995). They suggest that although faecal material is a suitable alternative to rumen liquor, separate calibrations with *in vivo* data are required as the rumen fluid and faecal inoculum produced slightly different gas production profiles.

Numerous *in vivo* and *in vitro* techniques are available for studying digestion and the nutritive quality of feedstuffs in ruminants (Johnson, 1966; section 2.2). Compared to ruminants, far fewer studies have been conducted with horses, yet feed represents approximately 60 to 70% of the cost associated with keeping horses (Cunha, 1980). Increased nutritive demands placed upon equines in relation to reproduction, growth, recreational / sporting activities and draught work means that it is important to feed a balanced ration which will enable the horse to perform to its full potential. Consequently, routine prediction of the nutritive value of feeds is important when devising rations for equines.

The prediction of OMD for equine feed values has been reported using the following predictor variables; chemical composition of feedstuffs (Martin-Rosset *et al.*, 1996a), a pepsin cellulase method (Martin-Rosset *et al.*, 1996b) and near infra-red reflectance spectrophotometry (Andrieu *et al.*, 1996 a and b). All of these studies derived prediction equations for 52 forages with known OMD and produced equations with R^2 values ranging from 0.878 to 0.931. Fonnesbeck (1981) has also published equations to predict the DE of equine feeds from their chemical composition. These

equations had R^2 values which ranged from 0.754 to 0.800, and were adopted by NRC (1989) to predict the DE values which appear in their tables of nutritive values for equine feeds.

In the current study the best single predictor of both DMIV and DE was the cumulative gas volume recorded at 44.5 h incubation (g44). In addition, the SMLR procedure selected g44 as the first parameter in all equations which incorporated cumulative gas volumes as predictor variables (equations 8.2, 8.3, 8.4, 8.11, 8.12, 8.13, 8.14 and 8.21). g44 is likely to be a function of both the rate and extent of feed degradation and hence gas production during incubation of feedstuffs. Equations selected from modelled gas production parameters (equations 8.5, 8.6, 8.7, 8.8, 8.15, 8.16 and 8.17) also contain predictor variables predominately reflecting the rate and extent of feed degradation (A, FRGP, Q, Z and t_{50}) although T was also included in equation 8.17.

Macheboeuf *et al.* (1997) have also reported the usefulness of cumulative gas volumes to predict *in vivo* parameters. Using caecal fluid as the source of inoculum, they found that the cumulative gas volume at 24 h incubation along with the CP content of the feed predicted the % OMD of grass forages and hays with an R^2 value of 0.87 and an RSD of 2.7. They also reported that the cumulative gas volume at 24 h could be used alone to predict OMD of alfalfa hays with an R^2 value of 0.76 and an RSD of 2.2. In the same study both the rate and extent of gas production were found to correlate well with OMD in alfalfa hays whilst the rate of gas production alone predicted OMD in grass forages and hays with an R^2 value of 0.74 and an RSD of 3.7. Similar findings were reported when equine faeces were used as the source of microbial inoculum during gas production studies with the same group of forages (Macheboeuf & Jestin, 1997).

Of the multiple linear regression equations derived in this study, those incorporating only VFA parameters as predictor variables had the highest R^2 and lowest RSD values for both DMIV (equation 8.9) and DE (equation 8.19). The total quantity of

VFA produced is likely to be a function of the extent of feed degradation whilst variations in Ac and Pr proportions probably reflect the starch and fibre contents of the individual feedstuffs. The use of VFA to predict the metabolisable energy content of ruminant feeds has been proposed by Dennison and Phillips (1983). They reported that cellulose digestion and VFA production were highly correlated (r = 0.88 - 0.99). The equations derived in the current study support their argument that VFA produced *in vitro* should provide a measure of the energy value of feedstuffs.

Although DM loss as a single predictor gave poor estimates of *in vivo* values, inclusion of DM loss in multiple regression equations did improve prediction of DE when included as a term with cumulative gas volumes (equation 8.14) and modelled gas production parameters (equation 8.17). However, in practical terms the determination of DM loss through filtration is time consuming and can cause problems when feedstuffs agglutinate during the incubation leading to poor filtration characteristics and thus highly variable results. Another potential source of error in DM loss measurements is the attachment of micro-organisms to feed particles leading to under estimation of DM loss (Theodorou *et al.*, 1995).

When all parameters were made available to the SMLR procedure, equations derived from sub-groupings of predictor variables were confirmed as having the highest R^2 and lowest RSD values (equations 8.3 & 8.9 for DMIV and equations 8.16, 8.17 and 8.19 for DE). Although additional highly significant relationships were found (equations 8.11, 8.12, 8.21, 8.22, 8.23 and 8.24) they generally did not improve the accuracy of either DMIV or DE prediction in terms of R^2 and RSD values. However, MSPE analysis identified systematic bias in equations 8.9, 8.11, 8.19, 8.21, 8.23 and 8.24 making them unsuitable for routine use. The identification of bias in equations 8.9 and 8.19 (the equations with the highest R^2 and lowest RSD values for DMIV and DE respectively) demonstrates the limitations of selecting predictive equations on the basis of their R^2 and RSD values alone.

The predictor variables used in this study increased in complexity from the cumulative gas volumes which require only the quantitative measurement of gas, through modelling gas production parameters which requires sophisticated mathematical analysis of data (France *et al.*, 1993), to measuring DM loss and VFA production requiring filtration and gas chromatography respectively. The range of analytical methodologies utilised in this evaluation therefore lend themselves to various experimental environments ranging from the simplest research station to more sophisticated laboratories. One of the original aims of this *in vitro* gas production technique (Theodorou *et al.*, 1994) was to provide a simple analytical tool for feed evaluation studies which could be employed in a range of laboratories world-wide. This study has confirmed the suitability of simple cumulative gas volumes or modelled gas production parameters as predictors of *in vivo* nutritive feed values for equines and demonstrated the widespread applicability of this *in vitro* batch culture technique for routine feed evaluation.

The prediction equations developed in this study correlated well with *in vivo* apparent digestibility and DE measurements suggesting that the pressure transducer technique along with associated DM loss and VFA analysis has considerable potential as a routine predictor of nutritive value for a wide range of equine feeds. However, a larger number of feedstuffs should be evaluated *in vitro* and *in vivo* to expand the database and thereby improve the accuracy and reliability of the derived prediction equations. Expansion of the database to allow the development of separate equations for forages and concentrates may also be necessary to increase the accuracy of predictions. It is also important to note that multiple linear regression equations of the type devised in this study may be unstable as predictive models when used with independent data sets due to multi-collinearity among predictor variables. Therefore more robust statistical methods such as principal component, ridge regression or partial least squares analysis should be used to derive predictive equations, thereby improving the accuracy and reliability of predictive equations.

8.2 Use of an automated gas production technique for evaluating the effect of antibiotics on the rumen microbial population.

8.2.1 Introduction

This chapter set out to investigate the potential use of the automated pressure evaluation system (APES; Davies *et al.*, 1995) for evaluating the effect of antibiotics on rumen fermentation *in vitro*. A brief introduction to the use of antibiotics in animal feeds is provided with a detailed description of the antibiotics selected for this study; monensin, avoparcin, penicillin G and chloramphenicol. The APES is described in chapter 2, section 2.1.4.3.2.

8.2.1.1 Antibiotics in animal feeds

Antibiotics have been used as feed supplements for farm animals (pigs, poultry and ruminants) for approximately forty years (Huntington, 1994; Nagaraja *et al.*, 1997). Their use as feed additives originates from investigations into the use of *Streptomyces aureofaciens* as a source of vitamin B_{12} for chickens. Supplementation with *S. aureofaciens* was seen to produce an increased growth rate in chicks even when the basal diet contained adequate amounts of vitamin B_{12}. This increase in growth rate was later attributed to the presence of small amounts of aureomycin in the feed and the benefits of aureomycin supplementation were investigated in other species of farm animal (Jukes and Williams, 1953). Since this time, several different antibiotics have been investigated for their ability to manipulate digestive processes, in pigs, poultry and ruminants. In this thesis antibiotics are discussed in relation to ruminants only.

The antibiotics used as feed additives for ruminants can be broadly divided into two categories; ionophore and non-ionophore antibiotics. The name ionophore means 'ion bearer' and refers to the ability of these compounds to transport metal ions and protons across cell membranes (Pressman, 1968; Schelling, 1984). Ionophore antibiotics that have been used or investigated for use in ruminant feeds include monensin, lasalocid, laidlomycin, lysocellin, narasin, salinomycin and tetronasin

(Bergen & Bates, 1984; Nagaraja *et al.*, 1997). Non-ionophore antibiotics represent a diverse group of compounds which includes avoparcin, bacitracins, chlortetracycline, flavomycin, neomycin, oxytetracycline, spiramycin, tylosin and virginiamycin (Nagaraja *et al.*, 1997).

8.2.1.2 The benefits of antibiotic supplementation

The benefit of antibiotic supplementation in ruminant feeds can be considered under two main categories (1) increased feed efficiency and (2) the prevention of ruminal disorders, such as, acidosis, bloat and acute bovine pulmonary oedema and emphysema (Muir & Barreto, 1979; Bergen & Bates, 1984; Nagaraja *et al.*, 1987; Lowe *et al.*, 1991; Underwood, 1992).

8.2.1.2.1 Increased feed efficiency

Although antibiotics have been shown to improve feed efficiency, their exact modes of action, are not well understood and are likely to differ with different antibiotics. It would appear that most of the antibiotics used as feed supplements for ruminants improve feed efficiency by manipulating the rumen fermentation; increasing propionate production and decreasing methane production (Nagaraja *et al.*, 1987; Zinn, 1993). The increase in propionate is generally accompanied by a decrease in acetate and butyrate, hence total VFA production remains unaltered (Richardson *et al.*, 1976; Bartley *et al.*, 1979; Bergen & Bates, 1984; Nagaraja *et al.*, 1987; Bogaert *et al.*, 1989). However, both increases and decreases in total VFA production have also been reported with antibiotic supplementation (Beede & Farlin, 1977; Baldwin *et al.*, 1982; van Nevel & Demeyer, 1992; Hino *et al.*, 1993). Increased propionate production leads to a more efficient fermentation as less energy is wasted in the production of gas, compared with the formation of acetate or butyrate where 3 or 4 moles of gas may be produced for every mole of acetate or butyrate formed (Hungate, 1966; Wolin & Miller, 1983). Propionate is also an important precursor of glucose synthesis and hence affects hormonal release and the distribution of nutrients (Elliot, 1980; Reynolds *et al.*, 1989).

A reduction in the amount of methane produced during fermentation as a result of antibiotic supplementation has been reported by several authors (Joyner *et al.*, 1979; Thornton & Owens, 1981; Mbanzamihigo *et al.*, 1996). This is advantageous in terms of both animal production, less energy is lost in the formation of waste gases, and from an ecological viewpoint, in terms of reduced production of green house gases. There are two possible mechanisms by which methane production can be reduced by antibiotic supplementation; (1) direct inhibition of the methanogenic micro-organisms themselves, and / or (2) a reduction in the availability of methanogenic precursors (CO_2, H_2 and formate). The rumen methanogens appear to be fairly resistant to both ionophore and non-ionophore antibiotics, therefore the reduction in methane production, seen when these antibiotics are fed, is thought to be due to a reduction in the availability of precursors (Chen & Wolin, 1979; Zinn, 1993).

Degradation of peptides and amino acids by rumen micro-organisms leads to losses in energy and nitrogen, as they are broken down and resynthesised into microbial protein before becoming available to the host animal, as opposed to passing to the abomasum and small intestine and being used by the host directly. Antibiotics may, therefore, also improve feed efficiency by altering the site of digestion. For example, ionophores can reduce ruminal degradation of peptides and deamination of amino acids allowing more efficient utilisation of these substances in the abomasum and small intestine (Hanson & Klopfenstein, 1979; Poos *et al.*, 1979; Bergen & Bates, 1984; Orskov, 1992).

8.2.1.2.2 Prevention of ruminal disorders

8.2.1.2.2.1 Lactic acidosis

Lactic acidosis is characterised by a low ruminal pH (5.6 in cases of chronic acidosis and 5.2 in acute acidosis) and accumulation of lactic acid (the normal concentration of lactic acid in the rumen is less than 1 mM, however in cases of acidosis the concentration can be greater than 100 mM) (Wiryawan & Brooker, 1995; Owens *et al.*, 1998). Lactic acidosis is often a problem in ruminants fed high grain diets,

especially when subjected to an abrupt increase in the proportion of concentrate in the diet and is initiated by the rapid proliferation of the Gram positive bacteria, *Streptococcus bovis*. This micro-organism grows rapidly on fermentable carbohydrate, with a doubling time of approximately 14 min (Russell & Hespell, 1981), and therefore rapidly ferments the carbohydrate to lactic acid (Muir & Barreto, 1979). As a result of the decrease in ruminal pH caused by the activity of *Streptococcus bovis*, rapid proliferation of acid tolerant *Lactobacillus* spp., ensues (Muir & Barreto, 1979). In addition, the lactic acid utilising bacteria are sensitive to low pH, and this prevents removal of the lactic acid and the rate of lactic acid production greatly exceeds the rate of utilisation. This abnormal production and accumulation of lactic acid leads to ruminal acidosis, which destroys the normal microbial population and produces potentially toxic metabolites. As many of the antibiotics used as feed supplements are selective for Gram positive bacteria, they can inhibit the major lactic acid producing bacteria (*S. bovis* and *Lactobacillus* spp.) whilst the Gram negative, lactic acid fermenting bacteria remain unaffected (see sections 8.1.4, 8.15, 8.1.6 and 8.1.7 for details of the mechanisms involved in bacterail inhibition by monenesin, avoparcin, penicillin G and chloramphenicol, respectively)(Dennis *et al.*, 1981; Newbold & Wallace, 1988).

8.2.1.2.2.2 Bloat

Bloat is a condition which is characterised by excessive foaming of the ruminal contents. It is often associated with acidodsis but can also occur when the pH of the rumen fluid is above 6.0 (Cheng *et al.*, 1998). There are two types of bloat (1) pasture bloat (also known as legume bloat), which occurs when animals consume high levels of legumes, such as lucerne (alfalfa) or clover and (2) grain bloat, which occurs when animals are fed high levels of rapidly fermentable cereals (Hungate, 1966; Clarke & Hungate, 1971; Bartley *et al.*, 1975; Reid *et al.*, 1975; McDonald *et al.*, 1995). Bloat occurs as a result of interactions between both the feed and the rumen micro-organisms (Reid *et al.*, 1975). *Lachnospira multipara* has been found in large numbers in steers fed legume based diets and is therefore thought to be involved in pasture bloat (Bryant *et al.*, 1961). However, the plant components may be the

primary causative agent of the foam in pasture bloat (Majak *et al.*, 1995). Whilst, microbial factors, such as excessive production of mucopolysaccharides (slime), appear to be the primary cause for the excessive foaming associated with grain bloat (Gutierrez *et al.*, 1961; Bartley *et al.*, 1975; Cheng *et al.*, 1976). For example, *Streptococcus bovis* is found in large numbers in animals suffering from grain bloat, and the production of slime from *S. bovis* is known to increase with an increase in the availability of energy (Cheng *et al.*, 1998). Although the number and type of bacteria and protozoa involved in bloat may differ with individual animals (Bryant *et al.*, 1961), ionophore supplementation (especially monensin or lasalocid) has been shown to reduce the incidence of bloat (Bartley *et al.*, 1983). The ionophores are effective in reducing the incidence of bloat through their antibacterial and antiprotozoal effects thereby preventing rapid proliferation of the causative micro-organisms.

8.2.1.2.2.3 Acute bovine pulmonary oedema and emphysema (ABPE)

Acute bovine pulmonary oedema and emphysema (ABPE) tend to occur when cattle are subjected to an abrupt change from poor to improved grazing or feeding conditions, for example when cattle are moved from dry, sparse grazing to lush green pastures. The pathology of the condition involves ruminal fermentation of tryptophan to 3-methylindole which causes lung damage (Carlson & Breeze, 1984). Firstly, the tryptophan is broken down into indoleacetic acid by a number of bacteria, such as *Escherichia coli*, *Pseudomonas fluorescens* and *Bacteroides fragilis* (Stowe, 1955; Chung *et al.*, 1975), these bacteria are not typical rumen bacteria but are probably enriched for by the substrate. Indoleacetic acid is then fermented by *Lactobacillus spp* to 3-methylindole (Carlson & Breeze, 1984). The ionophores, monensin and lasalocid help to prevent ABPE by inhibiting the Gram-positive bacteria thereby decreasing tryptophan degradation and preventing the accumulation of 3-methylindole (Carlson & Breeze, 1984; Nocerini *et al.*, 1985).

8.2.1.3 Evaluating the potential of antibiotics as feed supplements

Investigating the benefits of antibiotics is expensive to do *in vivo* as large numbers of animals and large quantities of feed and antibiotics are required. Preliminary *in vitro*

screening is therefore necessary to decide which antibiotics to look at in more detail *in vivo*. Several authors have investigated the effects of antibiotics on rumen fermentation *in vitro* using batch culture techniques (Beede & Farlin, 1977; Baldwin *et al.*, 1982; Nagaraja *et al.*, 1987; van Nevel & Demeyer, 1990 and 1992; Hino *et al.*, 1993). These techniques generally involve observations related to the effect on digestibility and provide end-point measurements. Gas production techniques, however, will allow the effects of antibiotics on the rate of fermentation to be investigated. *In vitro* continuous culture systems, such as RUSITEC, have also been used for investigating the effects of antibiotics (Stanier & Davies, 1981; Wallace *et al.*, 1981; Bogaert *et al.*, 1989). However 2 - 3 weeks (7 day adaptation period followed by 7 - 14 days recording period [Czerkawski & Breckenridge, 1977; Bogaert *et al.*, 1989]) are required to obtain results and the number of antibiotics which can be investigated at any one time is limited. Gas accumulation profiles obtained using batch culture gas production techniques can generally be obtained and collated within one week. In addition, several different antibiotics can be evaluated at any one time making it potentially more useful than continuous culture systems for screening antibiotics.

This study therefore set out to evaluate the APES for investigating the effects of antibiotic supplementation on rumen fermentation *in vitro* using monensin, avoparcin, penicillin G and chloramphenicol (Table 8.2.1). A brief description of each antibiotic follows.

8.2.1.4 Monensin

Monensin is an ionophore antibiotic which has been extensively studied both *in vivo* (Dinius et al., 1976; Hanson & Klopfenstein, 1979; Poos *et al.*, 1979; Thornton & Owens, 1981; Benz & Johnson, 1982; Goodrich *et al.*, 1984) and *in vitro* (van Nevel & Demeyer, 1977, 1990 and 1992; Beede & Farlin, 1977; Bartley *et al.*, 1979; Baldwin *et al.*, 1982; Nagaraja *et al.*, 1987; Callaway & Martin, 1996), making it a useful reference tool for this study. Monensin is produced by *Streptomyces cinnamonensis* and is active against Gram-positive bacteria, such as *Eubacterium,*

Table 8.2.1. Antibiotics used to evaluate the potential of an automated, *in vitro* gas production technique (APES) to investigate the effect of antibiotics on rumen fermentation.

Antibiotic	Chemical Formula	Producing organism	Primary antibacterial spectrum	Mode of action of bacterial inhibition
Monensin	$C_{36}H_{62}O_{11}$	*Streptomyces cinnamonensis*	Gram-positive	Disrupts the flow of cations across the cell membrane
Avoparcin	$C_{89}H_{101}ClN_9O_{36}$	*Streptomyces candidus*	Gram-positive	Inhibits cell wall synthesis
Penicillin G	$C_{16}H_{18}N_2SO_4$	*Penicillium notatum*	Gram-positive & certain Gram-negative	Inhibits cell wall synthesis
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	*Streptomyces venezuelae*	Gram-positive & Gram-negative	Inhibits protein synthesis

Lactobacillus and *Streptococcus* (Chen & Wolin, 1979) as well as those bacteria that often stain Gram-negative but have Gram-positive (i.e. gram-variable) cell wall structure such as *Butyrivibrio, Lachnospira* and *Ruminococcus*. Gram-negative bacteria, such as, *Bacteroides*, *Prevotella*, *Megasphaera*, *Selenomonas*, *Succinimonas*, *Succinivibrio* and *Veionella* species are resistant to monensin. Monensin is believed to inhibit Gram-positive bacteria by setting up an ion transport system across the cell membrane which results in the loss of intracellular potassium, accumulation of intracellular sodium and depletion of ATP (Bergen & Bates, 1984; Schelling, 1984). The Gram-negative bacteria are resistant as their outer membrane excludes monensin, the monensin molecules being too large to pass through the membrane (Nagaraja *et al.*, 1997). Monensin is also inhibitory to certain ruminal ciliates such as the entodiniomorphs (*Entodinium*, *Diplodinium* and *Ophryoscolex*) whilst the holotrichid ciliates, *Dasytricha*, *Isotricha* and *Charonina* tend to be resistant (Nagaraja *et al.*, 1997). Monensin can also be fungicidal at high concentrations (16 μg ml^{-1}) (Nagaraja *et al.*, 1997).

8.2.1.5 Avoparcin

Avoparcin is a glycopeptide produced by *Streptomyces candidus* (Nagaraja *et al.*, 1997). It inhibits the growth of Gram-positive bacteria, such as *Eubacterium* and *Streptococcus* as well as several gram-variable bacteria, *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, *R. flavefaciens* and *Lachnospira multiparus* (Stewart *et al.*, 1983). Avoparcin inhibits these bacteria by disrupting cell wall synthesis. This is achieved by inhibiting the incorporation of N-acetylglucosamine during peptidoglycan synthesis. Due to the nature of their cell wall (i.e. the absence of peptidoglycans) Gram-negative bacteria are resistant to avoparcin. Like monensin, avoparcin has been extensively studied both *in vivo* (Johnson *et al.*, 1979; Froetschel *et al.*, 1983; Cuthbert *et al.*, 1984; Haimoud *et al.*, 1995 and 1996) and *in vitro* (Jouany & Thivend, 1986; Nagaraja *et al.*, 1987; van Nevel & Demeyer, 1990 and 1992) making it a useful tool for evaluating the potential of the APES for investigating the effect of different substances as modifiers of rumen fermentation.

8.2.1.6 Penicillin G

Penicillin G was the first of the penicillins developed for medicinal use and according to Churchill's Medical Dictionary (1989) is the most widely used form of penicillin. It is produced from the Ascomycetous fungus *Penicillium notatum*, and is active against many bacteria especially Gram-positive bacteria such as *Streptococci*, *Staphylococci* and *Clostridia*. Like avoparcin, penicillin interferes with cell wall synthesis in Gram-positive bacteria; inhibiting the final stages of the synthesis of peptidoglycan. It is also effective against certain Gram-variable bacteria such as *Ruminococci* and some fungi (Stedman's Medical Dictionary, 1990). However, the activity of penicillin in the rumen may be limited as according to Wiseman *et al.* (1960) and Muir and Barreto (1979) the rumen contains an active penicillinase which can destroy the activity of the antibiotic.

8.2.1.7 Chloramphenicol

Chloramphenicol is not commonly used in feed supplements for ruminants, however its effect on rumen fermentation *in vitro* has been reported by Baldwin *et al.* (1982). Chloramphenicol (2-2-dichloro-N-[2-hydroxy-1-(hydroxy-methyl)-2-(4-nitrophenyl)ethyl] acetamide) was originally produced by *Streptomyces venezuelae*, although it is now more commonly produced by chemical synthesis rather than by microbial fermentation (Pratt & Fekety, 1986; Churchill's Medical Dictionary, 1989). Chloramphenicol inhibits protein synthesis in anaerobic bacteria by binding irreversibly to a receptor site on the 50 S subunit of the bacterial ribosome thus interfering with the formation of new peptides by blocking the action of peptidyl transferase (Pratt & Fekety, 1986; Lederberg, 1992). Chloramphenicol inhibits all anaerobic bacteria *in vitro* (Pratt & Fekety, 1986; Orpin & Joblin, 1997) thus allowing fermentation by fungi, yeasts and protozoa to be investigated. Hence, chloramphenicol is widely used in microbiological studies and in rumen microbiology is effective in selecting for rumen fungi *in vitro* (M.K. Theodorou - personal communication).

8.2.1.8 The future of antibiotic supplements

Although antibiotic supplementation of ruminant feeds appears to be beneficial, their future use in animal production is questionable due to increasing concern over public health including concern about residues in milk and meat. There is also concern about the potential for the selection of antibiotic resistant bacteria (Mudd, 1997; Nagaraja *et al.*, 1997). For these reasons, the European Unions Standing Committee on Feedstuffs banned the use of avoparcin in animal feeds on April 1, 1997 in order to review its safety (Mudd, 1997). However, if APES can detect differences in ruminal fermentation upon the addition of different antibiotics it may also be a useful screening tool for investigating the benefits of alternative, non-antibiotic feed additives such as probiotics (Williams & Newbold, 1990), enzymes (Armstrong & Gilbert, 1991) and naturally occurring compounds such as *Yucca shidigera* extract (Wallace *et al.*, 1994).

8.2.2 Materials and Methods

Feedstuff

Naked oats (Appendix 1) were used to examine the effects of four antibiotics on rumen fermentation *in vitro*.

Antibiotics

The antibiotics were avoparcin (Analytical standard; Roche Products Ltd., Gosport, Hampshire, UK), monensin (monensin sodium salt RSO188; Dista Products Ltd., Speke, Liverpool, UK), penicillin G (Sigma Chemicals Company Ltd., Poole, Dorset, UK) and chloramphenicol (Sigma Chemicals Company Ltd., UK).

Antibiotic concentrations of 0, 1, 10 or 20 ppm in the rumen fluid, were used [in accordance with studies by Baldwin *et al.* (1982) and Van Nevel and Demeyer (1990)]. All antibiotics were dissolved in solution and prepared at different concentrations, such that 1 ml of antibiotic solution would give a concentration of 0, 1, 10 or 20 ppm in the rumen fluid. Avoparcin and penicillin G were dissolved in water; 100 mg of each antibiotic was dissolved in 200 ml of distilled water. This solution (2, 20 or 40 ml) was then made up to 100 ml with distilled water, giving

antibiotic concentrations of 1, 10 or 20 ppm in the rumen fluid (when 1 ml of the solution was injected into the fermentation bottles). Monensin and chloramphenicol solutions were prepared using a 50:50 methanol:distilled water mixture, as these antibiotics are only slightly soluble in water. Monensin or chloramphenicol (100 mg) were weighed out into a 100 ml volumetric flask. Methanol was then added to give 100 ml of solution. This solution was transferred into a 200 ml volumetric flask and distilled water added to give 200 ml of antibiotic solution. Aliquots (2, 20 or 40 ml) of this solution were then made up to 100 ml with 50:50 methanol:distilled water giving concentrations of 1, 10 or 20 ppm in the rumen fluid when 1 ml of the respective solutions was injected into the fermentation bottle.

Experimental protocol

The automated pressure evaluation system (APES) (Davies *et al.*, 1995) was used to investigate the effects of four antibiotics on rumen fermentation *in vitro*.

Two antibiotics were investigated per experiment. 1.00 g DM naked oats was weighed into 24 x 150 ml Duran bottles (Fisher Scientific UK, Loughborough, UK; 3 replicates per antibiotic at each treatment level; 0, 1, 10 or 20 ppm). The remaining bottles contained no substrate and acted as control blanks for the different levels of antibiotic (Table 8.2.2). Bottles which received 0 ppm antibiotic were injected with 1 ml of the respective solvent at the same time as the antibiotics were administered. This procedure followed Beede and Farlin (1977) who reported that 1 ml additions of distilled water or methanol had no effect on *in vitro* fermentation.

Medium (85 ml) was dispensed into each bottle and 4 ml reducing agent added (section 3.7). The bottles were sealed using screw top caps (Duran; Fisher Scientific UK) and stored at 4 °C for 9 h. The bottles were then warmed to 39 °C and attached to the APES by connecting each bottle to its own individual pressure sensor and solenoid valve.

Table 8.2.2 Experimental protocol for evaluating the potential of an automated gas production technique (Davies *et al.*, 1995) for investigating the effect of antibiotics on rumen fermentation.

Bottle no.	Substrate [1]	Antibiotic [2]		Antibiotic concentration [3]
		Experiment 8.2.1	Experiment 8.2.2	
1 - 3	+			0
4 - 6	+	avoparcin	monensin	1
7 - 9	+	avoparcin	monensin	10
10 - 12	+	avoparcin	monensin	20
13 - 15	-			0
16 - 18	-	avoparcin	monensin	1
19 - 21	-	avoparcin	monensin	10
22 - 24	-	avoparcin	monensin	20
25 - 27	+	penicillin G	chloramphenicol	1
28 - 30	+	penicillin G	chloramphenicol	10
31 - 33	+	penicillin G	chloramphenicol	20
34 - 36	-	penicillin G	chloramphenicol	1
37 + 38	-	penicillin G	chloramphenicol	10
39 + 40	-	penicillin G	chloramphenicol	20

[1]The substrate was 1.00 g DM naked oats and was either present (+) or absent (-). [2]Antibiotics were dissolved in either distilled water (experiment 8.2.1) or a 50:50 methanol:distilled water mixture (experiment 8.2.2), and injected into fermentation bottles as 1 ml of solution, 2 h 40 min after inoculation. [3]The concentration of antibiotics was parts per million of rumen fluid inoculum. Blanks with 0 ppm antibiotic recieved 1 ml of the respective solvent.

Microbial inoculum was prepared from rumen fluid collected from a sheep fed hay *ad libitum* (section 3.4). The APES was switched on and each bottle was inoculated with 10 ml of inoculum. Avoparcin and penicillin G (experiment 8.2.1) or monensin and chloramphenicol (experiment 8.2.2) were prepared as described above, and injected into their respective bottles (Table 8.2.2) 2 h 40 min after inoculation with rumen micro-organisms.

When fermentations were complete, 72 h after inoculation, the APES was switched off and the bottles were removed from the system. The pH was recorded in all bottles and samples were taken for VFA and lactate analysis (section 3.12.1 and 3.12.2, respectively). The volume of culture fluid removed for VFA and lactate analysis (1.2 ml and 1 ml, respectively) was replaced with distilled water, and all bottles were returned to the APES in order that the vent volume could be calibrated. The vent volume is the volume of gas required to produce a pressure of 4.5 kPa, and hence vent the bottles. Calibration involved inserting a syringe needle, attached to two 2 ml syringes via a 3 way valve, through the cap of the bottle. Each syringe contained 2 ml gas (air) and this was slowly injected into the bottle until a vent was initiated. This was repeated three times and the mean volume of gas required to vent the bottle was noted. All forty bottles used in the experiment were calibrated, following both experiment 1 and experiment 2. After calibration, DM loss was determined as described in section 3.10.1.

The data for each bottle was imported into the spread sheet programme Quattro - Pro and the calibration volumes were used to construct cumulative gas production profiles for each bottle. Each experiment was a factorial design consisting of 7 treatments [2 antibiotics, 3 antibiotic concentrations (1, 10 or 20 ppm) and one set of control bottles (0 ppm antibiotic)] and 3 replicate bottles (7 x 3). The resulting gas production profiles were fitted to the model of France *et al.* (1993) (section 3.11) and analysed using parallel curve analysis (section 3.11). Values for the modelled gas production parameters, DM loss, VFA production, lactate production and pH were analysed using analysis of variance in Genstat 5 (Lawes Agricultural Trust, 1993). Differences between treatments were calculated using the LSD (section 3.11).

8.2.3 Results

8.2.3.1 Gas production profiles

Avoparcin and Penicillin G

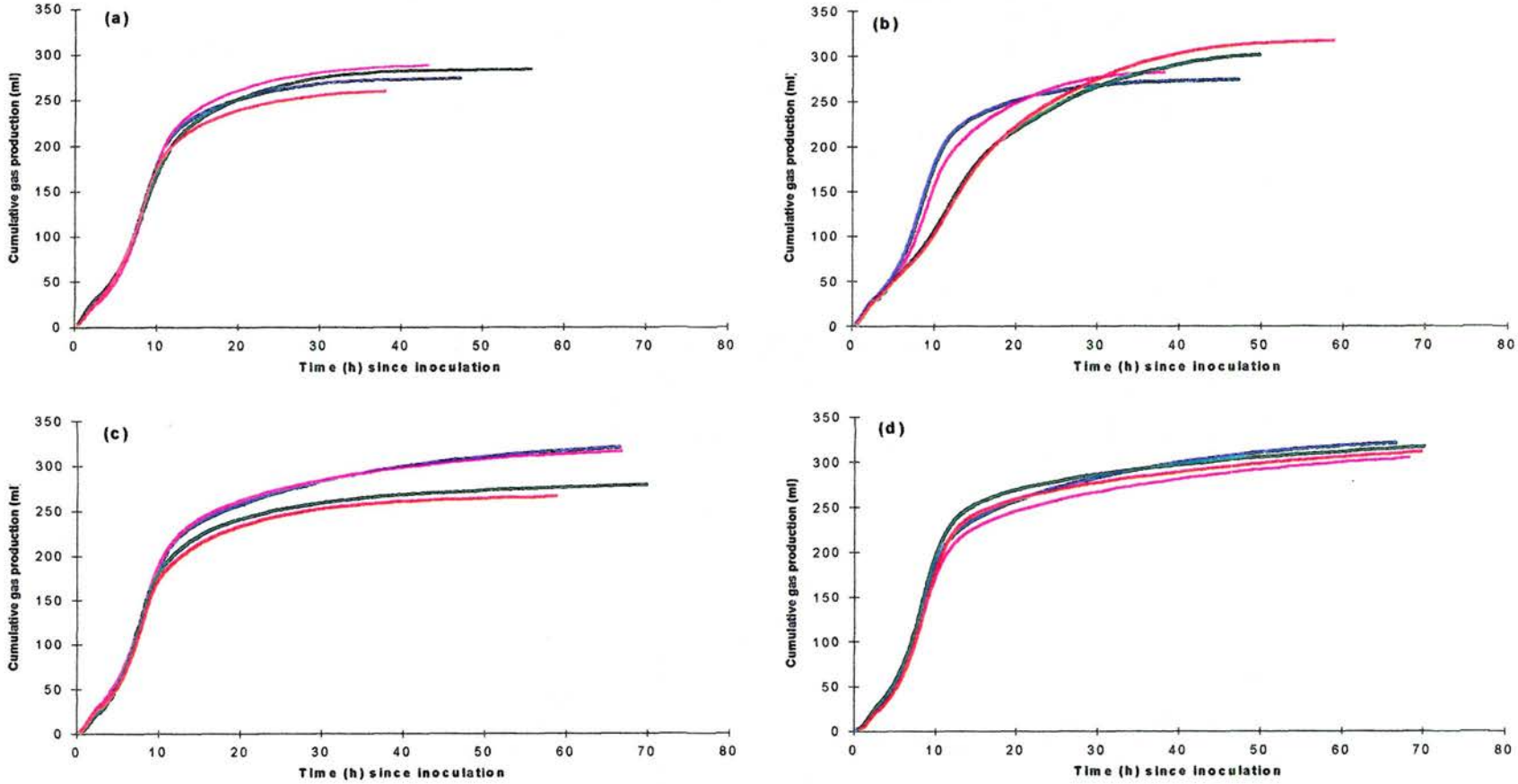
The gas production profiles obtained during incubation of 1.00 g naked oats with avoparcin or penicillin G at concentrations of 0, 1, 10 or 20 ppm are shown in

Figures 8.2.1.a and b, respectively. In all gas production profiles, a negligible effect on gas production was observed at 2 h 40 min, corresponding to the injection of the antibiotics (Figure 8.2.2). Occasionally, however, there was a trend for reduced gas production, for approximately 20 min after injection of the antibiotic, but this was negligible relative to the total gas production profiles.

Parallel curve analysis indicated no significant differences between the rate and total volume of gas produced during incubation with 0, 1, 10 or 20 ppm avoparcin, with the exception of 1 ppm avoparcin which produced significantly more gas than incubation with 20 ppm avoparcin ($p < 0.01$; Appendix 8.2.1.1). The addition of 1, 10 and 20 ppm penicillin G to the incubation resulted in significantly different gas production profiles compared to the control bottles in terms of both the rate and total volume of gas produced ($p < 0.01$; Apendix 8.2.1.2). There were also significant differences between the different concentrations of penicillin G; incubation with 1 ppm producing gas significantly faster but with a lower total volume of gas than bottles containing either 10 or 20 ppm ($p < 0.001$; Appendix 8.2.1.2). Parallel curve analysis also indicated significant differences in the rate and total volume of gas produced between bottles incubated with avoparcin or penicillin G at all concentrations. However, there were the following exceptions; similar rates of gas production were seen between 1 ppm avoparcin and 1 ppm penicillin G and between 10 ppm avoparcin and 1 ppm penicillin G, whilst similar volumes of gas were produced after incubation with 20 ppm avoparcin and 1 ppm penicillin G (Appendix 8.2.1.3).

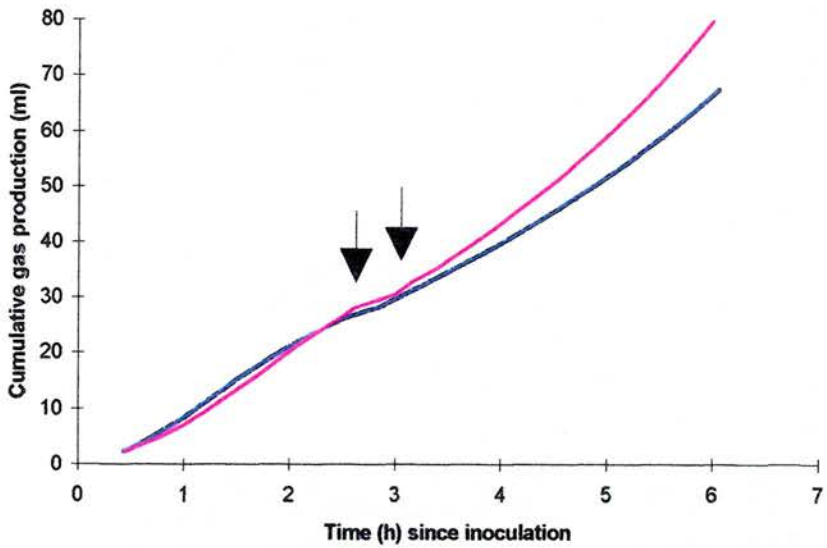
The fitted parameters and derived quantities for the gas production profiles obtained during incubation with avoparcin and penicillin G are shown in Table 8.2.3. The rates of gas production, b and c, ranged from 0.1116 (during incubation with 10 ppm penicillin G) to 0.2786 h^{-1} (for the control bottles, 0 ppm antibiotic) and from -0.780 (for the control bottles) to -0.273 $h^{-0.5}$ (during incubation with 10 ppm penicillin G) for b and c respectively. The mean values of b and c were 0.2165 ± 0.02577 h^{-1} and -0.5896 ± 0.07136 $h^{-0.5}$. The total cumulative gas production, A, was greatest during

Figure 8.2.1 Cumulative gas production profiles obtained during the incubation of 1.00 g naked oats (*Avena nuda*) with 0 (blue), 1 (pink), 10 (green) or 20 (red) ppm of either (**a**) avoparcin, (**b**) penicillin G, (**c**) monensin or (**d**) chloramphenicol.



Each line represents the mean of three bottles described by the model of France *et al.* (1993). Fermentations were conducted using the APES (Davies *et al.*, 1995), each bottle contained 1.00 g naked oats, 89 ml culture medium and 10 ml of a rumen microbial inoculum. Bottles were injected with 0, 1, 10 or 20 ppm avoparcin, penicillin G, monensin or chloramphenicol 2 h 40 min after inoculation.

Figure 8.2.2 Depression in the cumulative gas production profile from 1.00 g naked oats incubated with a rumen microbial inoculum corresponding to the addition of 1 ml of water (blue) or 1 ml 50:50 water:methanol (pink).



Fermentations were conducted using the APES, each bottle contained 1.00 g naked oats, 89 ml culture medium and 10 ml rumen microbial inoculum. Similar depressions in gas production were observed when 1 ml water containing 1, 10 or 20 ppm avoparcin or penicillin G and 1 ml water:methanol containing 1, 10 or 20 ppm monensin or chloramphenicol were added to the fermentation.

Table 8.2.3 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and treated with 0, 1, 10 and 20 ppm of avoparcin or penicillin G.

Gas production parameters	Control (0 ppm antibiotic)	Avoparcin (ppm)			Penicillin G (ppm)			s.e.d
		1	10	20	1	10	20	
b (h^{-1})	0.2786^{a}	0.2524^{a}	0.2468^{a}	0.2698^{a}	0.2285^{a}	0.1116^{b}	0.1279^{b}	0.0262
c ($h^{-0.5}$)	-0.780^{a}	-0.698^{a}	-0.666^{a}	-0.700^{a}	-0.634^{a}	-0.273^{b}	-0.376^{b}	0.1003
A (ml)	$273.8^{a,b}$	283.0^{b}	281.0^{b}	259.5^{a}	285.2^{b}	306.1^{c}	313.2^{c}	6.77
B	156.8^{a}	173.5^{a}	176.1^{a}	160.1^{a}	179.3^{a}	249.0^{b}	226.8^{b}	18.11
L_T (h)	$1.95^{a,b}$	$1.86^{a,c}$	$1.80^{a,c}$	$1.68^{b,c}$	$1.92^{a,b}$	1.49^{c}	2.12^{a}	0.1895
t50 (h)	$8.76^{a,b}$	$9.13^{a,c}$	8.98^{a}	8.22^{b}	9.61^{c}	13.27^{d}	13.89^{e}	0.245
t95 (h)	21.81^{a}	$23.65^{a,b}$	$23.32^{a,b}$	21.31^{a}	25.03^{b}	40.60^{c}	40.06^{c}	1.435

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 8.2.1.a. and Figure 8.2.1.b. Values in rows not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables for the above data are shown in Appendix 8.2.1.4 - 8.2.1.10. Parallel curve analysis of the data is shown in Appendix 8.2.1.1 - 8.2.1.3.

incubation with 20 ppm penicillin G (313.2 ml), whilst least gas was produced during incubation with 20 ppm avoparcin (259.5 ml), with a mean value across all treatments of 286.0 ± 6.95 ml. The lag time, L_T, ranged from 1.49 h (during incubation with 10 ppm penicillin G) to 2.12 h (during incubation with 20 ppm penicillin G), with a mean value of 1.83 ± 0.0766 h. The time taken to produce 50 and 95 % of the total gas production (t_{50} and t_{95}, respectively) were similar between control bottles and all concentrations of avoparcin whilst an increased concentration of penicillin G generally increased t_{50} and t_{95}. Values for t_{50} ranged from 8.22 (during incubation with 20 ppm avoparcin) to 13.89 h (during incubation with 20 ppm penicillin G), with a mean value of 10.26 ± 0.873 h. Whilst t_{95} ranged from 21.31 h (during incubation with 20 ppm avoparcin) to 40.60 h (during incubation with 10 ppm penicillin G), with a mean value of 27.97 ± 3.225 h.

Monensin and chloramphenicol

The gas production profiles obtained during incubation of 1.00 g DM naked oats with 0, 1, 10 or 20 ppm monensin or chloramphenicol are shown in Figures 8.2.1.c. and d, respectively. As for avoparcin and penicillin G (dissolved in water), injection of 0, 1, 10 or 20 ppm monensin or chloramphenicol (dissolved in 50:50 water : methanol) had a negligible effect on gas production at 2 h 40 min, corresponding to the injection of the antibiotics (Figure 8.2.2). Occasionally, however, there was a trend for reduced gas production, for approximately 20 min after injection of the antibiotic, but this was negligible relative to the total gas production profiles.

Parallel curve analysis of the gas production profiles obtained during incubation with monensin indicated significantly different rates of gas production between 1 and 10 ppm monensin and significantly different total gas production between control bottles (0 ppm antibiotic) and both 10 and 20 ppm monensin ($p < 0.001$) and between 1 ppm and both 10 and 20 ppm monensin ($p < 0.001$; Appendix 8.2.2.1). No significant differences in the rate or total volume of gas produced were seen between the different concentrations of chloramphenicol with the following exceptions; (1) 0 and 1 ppm chloramphenicol, (2) 1 and 10 ppm chloramphenicol and (3) 10 and 20 ppm

chloramphenicol, which all resulted in the production of significantly different volumes of gas ($p < 0.05$; Appendix 8.2.2.2). Parallel curve analysis between all concentrations of the different antibiotics showed no significant differences between the gas production profiles for 1 ppm monensin and 10 ppm chloramphenicol, whilst all other combinations resulted in significant differences between the rate of gas production and / or the total volume of gas produced ($p < 0.05$; Appendix 8.2.2.3).

The fitted parameters and derived quantities for the gas production profiles are shown in Table 8.2.4. The rates of gas production, b and c, ranged from 0.2403 h^{-1} (during incubation with 1 ppm monensin) to 0.3121 h^{-1} (during incubation with 10 ppm monensin) and from -0.910 $h^{-0.5}$ (during incubation with 20 ppm chloramphenicol) to -0.623 $h^{-0.5}$ (during incubation with 1 ppm monensin), for b and c respectively. The mean values of b and c were 0.2833 ± 0.01023 h^{-1} and -0.8017 ± 0.04327 $h^{-0.5}$. The total cumulative gas production, A, was similar between control bottles and bottles inoculated with all concentrations of chloramphenicol, whilst concentrations of 10 and 20 ppm monensin significantly reduced the total gas production relative to control bottles ($p < 0.05$). Values for A ranged from 252.3 ml (during incubation with 20 ppm monensin) to 303.7 ml (during incubation with 10 ppm chloramphenicol), with a mean value across all treatments of 284.0 ± 6.97 ml. The lag time, L_T, was not affected by the addition of monensin but increased significantly with the addition of chloramphenicol. Values for L_T ranged from 1.68 h (during incubation with 1 ppm monensin) to 2.26 h (during incubation with 20 ppm chloramphenicol), with a mean value of 1.97 ± 0.088 h. The time taken to produce 50 and 95 % of the total gas production (t_{50} and t_{95}, respectively) ranged from 8.29 h (during incubation with 20 ppm monensin) to 9.05 h (for control bottles) and from 20.41 h (during incubation with 10 ppm monensin) to 23.16 h (during incubation with 1 ppm monensin), respectively. The mean value for t_{50} was 8.73 ± 0.110 h whilst the mean for t_{95} was 21.75 ± 0.379 h.

Table 8.2.4 The gas production parameters; rates of degradation (b and c), asymptote of gas production (A), B, lag time (L_T), and time taken to produce 50 or 95 % of the gas production (t_{50} and t_{95}, respectively) for 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and treated with 0, 1, 10 and 20 ppm of monensin or chloramphenicol.

Gas production parameters	Control (0 ppm antibiotic)	Monensin (ppm) 1	Monensin (ppm) 10	Monensin (ppm) 20	Chloramphenicol (ppm) 1	Chloramphenicol (ppm) 10	Chloramphenicol (ppm) 20	s.e.d
b (h^{-1})	$0.2538^{a,b}$	0.2403^{a}	0.3121^{c}	$0.2787^{b,d}$	$0.2944^{c,d}$	$0.3019^{c,d}$	$0.3022^{c,d}$	0.01406
c ($h^{-0.5}$)	-0.694^{a}	-0.623^{a}	-0.893^{b}	$-0.740^{a,c}$	$-0.866^{b,c}$	-0.886^{b}	-0.910^{b}	0.0632
A (ml)	299.4^{a}	$286.2^{a,b}$	$266.1^{b,c}$	252.3^{c}	$288.2^{a,b}$	303.7^{a}	292.4^{a}	12.03
B	$183.8^{a,b}$	187.7^{a}	138.5^{c}	151.0^{c}	149.9^{c}	$155.6^{b,c}$	144.4^{c}	13.58
L_T (h)	$1.78^{a,b}$	1.68^{a}	$2.03^{b,c}$	$1.76^{a,b}$	2.16^{c}	2.15^{c}	2.26^{c}	0.1334
t50 (h)	9.05^{a}	$8.79^{a,b,c}$	$8.38^{b,c}$	8.29^{c}	$8.90^{a,b}$	$8.77^{a,c}$	8.98^{a}	0.2667
t95 (h)	23.04^{a}	23.16^{a}	20.41^{b}	$21.21^{b,c}$	21.66^{c}	$21.23^{b,c}$	$21.57^{b,c}$	0.543

Values shown in the table were obtained by fitting the model of France *et al.* (1993) to the gas production profiles shown in Figure 8.2.1.c. and Figure 8.2.1.d. Values in rows not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance tables for the above data are shown in Appendix 8.2.2.4 - 8.2.2.10. Parallel curve analysis of the data is shown in Appendix 8.2.2.1 - 8.2.2.3.

8.2.3.2 Dry matter loss

Avoparcin and penicillin G

Incubation of naked oats with 1, 10 or 20 ppm avoparcin had no significant effect on the loss of DM compared to controls (0 ppm avoparcin), with an average value of 923.9 ± 1.94 mg g^{-1} being degraded by 72 h incubation in all cases (Table 8.2.5). Increasing the concentration of penicillin G during the incubation of naked oats, resulted in a significant decrease in DM loss ($p < 0.05$). Concentrations of 10 and 20 ppm penicillin G significantly reduced the loss of DM from naked oats compared to incubation without penicillin G (Table 8.2.5).

Table 8.2.5 Dry matter lost (mg g^{-1}) from 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 and 20 ppm of avoparcin or penicillin G.

Control	Avoparcin (ppm)			Penicillin G (ppm)		
(0 ppm)	1	10	20	1	10	20
919.6^{a}	925.1^{a}	922.4^{a}	928.7^{a}	$907.9^{a,b}$	$882.0^{b,c}$	864.2^{c}

Values not bearing the same superscripts differ significantly ($p < 0.05$), sed = 16.83. The analysis of variance table is shown in Appendix 8.2.3.

Monensin and chloramphenicol

Incubation of naked oats with 1, 10 or 20 ppm monensin or chloramphenicol had no significant effect on the loss of DM compared to controls (0 ppm monensin / chloramphenciol), with a mean value for DM loss across all monensin treatments of 919.1 ± 2.47 mg g^{-1} and across all chloramphenicol treatments the mean value was 928.7 ± 3.09 mg g^{-1}. (Table 8.2.6).

Table 8.2.6 Dry matter lost (mg g^{-1}) from 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 and 20 ppm of monensin or chloramphenicol.

Control	Monensin (ppm)			Chloramphenicol (ppm)		
(0 ppm)	1	10	20	1	10	20
$923.8^{a,b}$	913.0^{a}	$922.3^{a,b}$	917.1^{a}	$927.6^{a,b}$	937.7^{b}	$925.8^{a,b}$

Values not bearing the same superscripts differ significantly ($p < 0.05$), sed = 9.27. The analysis of variance table is shown in Appendix 8.2.4.

8.2.3.3 Volatile fatty acid (VFA) production

Avoparcin and penicillin G

VFA production during incubation of naked oats with 1, 10 and 20 ppm of avoparcin and penicillin G is shown in Table 8.2.7. There was no significant difference in the total quantity of VFA produced between the bottles containing different concentrations of avoparcin and the control bottles at the end of the incubation. However for penicillin G bottles containing 1 or 10 ppm penicillin G produced significantly less total VFA than the control bottles ($p < 0.05$).

The molar percentages of the individual acids were also seen to vary between treatments (Table 8.2.7). The percentage of acetate produced was similar between control bottles and those containing all concentrations of avoparcin, whilst the percentage of acetate produced after addition of penicillin G significantly increased with the addition of 10 or 20 ppm ($p < 0.05$). The percentage of propionate produced during incubation with 1 ppm avoparcin was similar to that produced in control bottles, whilst all other concentration of avoparcin and 1 ppm penicillin G produced significantly higher percentages of propionate than controls ($p < 0.05$). For 10 and 20 ppm penicillin G the percentage of propionate was significantly less than that produced in control bottles ($p < 0.05$). The percentage of butyrate produced during incubation with 1 ppm avoparcin was also similar to that produced in control bottles, whilst the other concentrations of avoparcin and all concentrations of penicillin G resulted in significantly lower percentages of butyrate than control bottles ($p < 0.05$). The percentage of valerate produced was similar for control bottles and both

Table 8.2.7 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced during incubation of naked oats (*Avena nuda*) with a rumen microbial inoculum and either avoparcin or penicillin G at a concentration of 0, 1, 10 or 20 ppm.

VFA	Control (0 ppm antibiotic)	Avoparcin (ppm)			Penicillin G (ppm)			sed
		1	10	20	1	10	20	
Total (mmol l^{-1})	77.03^{a}	77.41^{a}	76.66^{a}	$74.13^{a,b}$	68.69^{b}	68.60^{b}	$73.86^{a,b}$	2.676
Acetate (molar %)	$46.79^{a,b}$	46.43^{a}	$46.64^{a,b}$	$46.99^{a,b}$	47.64^{b}	57.25^{c}	62.95^{d}	0.523
Propionate (molar %)	35.77^{a}	36.62^{a}	40.61^{b}	43.07^{c}	40.32^{b}	30.01^{d}	21.77^{e}	0.880
Butyrate (molar %)	13.87^{a}	13.47^{a}	9.97^{b}	7.15^{c}	$8.35^{b,c}$	9.86^{b}	9.97^{b}	0.868
Valerate (molar %)	3.58^{a}	3.48^{a}	2.77^{a}	2.79^{a}	3.70^{a}	2.87^{a}	5.30^{b}	0.632

Values in rows not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance table is shown in Appendix 8.2.5.1 - 8.2.5.5.

antibiotics, with the exception of incubation with 20 ppm penicillin G were the percentage of valerate was significantly higher than all other treatments ($p < 0.05$).

Monensin and chloramphenicol

No significant differences were observed in the total quantity of VFA produced after incubation with 1 or 10 ppm monensin, however a significant increase in the total quantity of VFA produced was measured in bottles which had been incubated with 20 ppm monensin ($p < 0.05$; Table 8.2.8). All concentrations of chloramphenicol produced similar quantities of total VFA as the control bottles.

The molar percentages of VFA produced also varied when naked oats were incubated with monensin and chloramphenicol (Table 8.2.8). The molar percentages of both acetate and butyrate produced during incubation with monensin and chloramphenicol were similar to that produced in control bottles. However, incubation with 20 ppm monensin resulted in a significantly higher percentage of acetate than incubation with 1 ppm monensin ($p < 0.05$). Whilst incubation with 10 and 20 ppm monensin produced a significantly lower percentage of butyrate than incubation with 1 ppm monensin ($p < 0.05$). The percentage of propionate was similar between control bottles and incubation with 1 ppm monensin, whilst incubation with 10 and 20 ppm monensin significantly increased the proportion of propionate compared to control bottles ($p < 0.05$). Similarly the percentage of propionate after incubation with 1 and 20 ppm chloramphenicol was similar to control bottles, whilst incubation with 10 ppm chloramphenicol resulted in a significantly higher percentage of propionate compared to controls ($p < 0.05$). The percentage of valerate also varied, with 1 and 10 ppm monensin producing higher percentages of valerate than controls ($p < 0.05$), whilst incubation with 10 ppm chloramphenicol produced significantly less valerate than controls.

Table 8.2.8 Total volatile fatty acid (VFA) production (mmol l^{-1}) and the molar percentages of the individual VFA; acetate, propionate, butyrate, and valerate produced during incubation of naked oats (*Avena nuda*) with a rumen microbial inoculum and either monensin or chloramphenicol at a concentration of 0, 1, 10 or 20 ppm.

VFA	Control (0 ppm antibiotic)	Monensin (ppm)			Chloramphenicol (ppm)			sed
		1	10	20	1	10	20	
Total (mmol l^{-1})	$61.34^{a,b}$	$61.22^{a,b}$	56.84^{b}	66.59^{c}	$63.40^{a,c}$	$65.14^{a,c}$	$61.42^{a,b}$	2.233
Acetate (molar %)	$41.98^{a,b}$	40.63^{a}	$41.29^{a,b}$	43.16^{b}	$41.39^{a,b}$	$41.45^{a,b}$	$41.34^{a,b}$	1.035
Propionate (molar %)	39.76^{a}	$41.24^{a,c}$	47.98^{b}	46.78^{b}	$40.75^{a,c}$	41.76^{c}	$40.90^{a,c}$	0.893
Butyrate (molar %)	$9.53^{a,b}$	13.87^{a}	6.55^{b}	6.12^{b}	14.28^{a}	13.77^{a}	14.07^{a}	2.444
Valerate (molar %)	3.62^{a}	4.26^{b}	4.18^{b}	3.94^{a}	3.58^{a}	3.17^{c}	3.69^{a}	0.165

Values in rows not bearing the same superscript differ significantly ($p < 0.05$). The analysis of variance table is shown in Appendix 8.2.6.1 - 8.2.6.5.

8.3.4 Production of lactic acid

Avoparcin and penicillin G

The concentration of L - lactate present at the end of the incubation was similar between control bottles and those incubated with 10 ppm avoparcin and 1 or 20 ppm penicillin G, whilst the concentration of L - lactate was significantly lower than in control bottles after incubation with 1 or 20 ppm avoparcin and 10 ppm penicillin G ($p < 0.05$; Table 8.2.9). Incubation with all concentrations of avoparcin and 10 ppm penicillin G resulted in significantly lower quantities of D - lactate than controls ($p < 0.05$). Whilst incubation with 1 or 20 ppm penicillin G produced a similar quantity of D-lactate as controls. Correspondingly there were differences in the total quantity of lactate present after incubation. All concentrations of avoparcin and 10 ppm penicillin G resulted in significantly lower quantities of lactate at the end of the incubation compared to controls ($p < 0.05$). Whilst incubation with 1 and 20 ppm penicillin G produced similar levels of lactate to control bottles.

Monensin and chloramphenicol

Incubation with all concentrations of monensin and chloramphenicol resulted in lower quantities of L - lactate than control bottles ($p < 0.05$; Table 8.2.10). For 10 ppm monensin, the quantity of D - lactate was significantly lower than that seen in control bottles ($p < 0.05$), whilst the remaining treatments resulted in similar levels of D - lactate as the control bottles. For chloramphenicol, D-lactate production was similar between control bottles and incubation with 1 and 20 ppm, whilst incubation with 10 ppm resulted in a significantly higher quantity of D-lactate than controls ($p < 0.05$). Due to the lower concentrations of L-lactate, the total quantity of lactate after incubation with all concentrations of monensin and chloramphenicol was significantly lower than in control bottles ($p < 0.05$).

Table 8.2.9 Lactic acid concentration (μg ml^{-1}) after incubation of naked oats (*Avena nuda*) with a rumen microbial inoculum in the presence of 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Lactic acid	Control (0 ppm antibiotic)	Avoparcin (ppm)			Penicillin G (ppm)			sed
		1	10	20	1	10	20	
L-lactate	29.3^{a}	$12.6^{b,c}$	$20.5^{a,b}$	1.8^{c}	29.7^{a}	$10.6^{b,c}$	$23.8^{a,b}$	5.92
D-lactate	$50.1^{a,b}$	$34.2^{c,d}$	$33.7^{c,d}$	30.9^{d}	56.8^{a}	$34.3^{c,d}$	$43.5^{b,c}$	4.98
Total	79.3^{a}	$46.8^{b,d}$	$54.2^{b,c}$	32.7^{d}	86.5^{a}	$44.9^{c,d}$	$67.3^{a,b}$	9.59

Values not bearing the same superscripts differ significantly ($p < 0.05$). The statistical analysis of the above values is shown in Appendix 8.2.7.1 - 8.2.7.3.

Table 8.2.10 Lactic acid concentration (μg ml^{-1}) after incubation of naked oats (*Avena nuda*) with a rumen microbial inoculum in the presence of 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Lactic acid	Control (0 ppm antibiotic)	Monensin (ppm) 1	Monensin (ppm) 10	Monensin (ppm) 20	Chloramphenicol (ppm) 1	Chloramphenicol (ppm) 10	Chloramphenicol (ppm) 20	sed
L-lactate	49.7^{a}	17.6^{b}	39.4^{c}	17.0^{b}	16.2^{b}	20.1^{b}	14.6^{b}	3.99
D-lactate	$31.8^{a,b}$	28.1^{a}	14.0^{c}	32.4^{a}	$39.7^{b,d}$	44.1^{d}	$36.2^{a,d}$	3.83
Total	81.5^{a}	45.7^{b}	$53.3^{b,c}$	49.4^{b}	$55.9^{b,c}$	64.2^{c}	$50.7^{b,c}$	6.52

Values not bearing the same superscripts differ significantly ($p < 0.05$). The statistical analysis of the above values is shown in Appendix 8.2.8.1 - 8.2.8.3.

8.2.3.5 Changes in batch culture pH

Avoparcin and penicillin G

There were no significant differences in the pH after incubation with 0, 1, 10, or 20 ppm avoparcin or penicillin G (Appendix 8.2.9). The mean pH value across all treatments was 6.4 ± 0.01 pH units.

Monensin and chloramphenicol

The pH after incubation with 10 or 20 ppm monensin and all concentrations of chloramphenicol was significantly lower than in control bottles (containing no monensin or chloramphenicol) and in bottles containing 1 ppm monensin ($p < 0.05$; Table 8.2.11; Appendix 8.2.10). Although statistically significant, this is unlikely to be biologically significant as the mean pH in control bottles was 6.5 compared to a pH of 6.3 in bottles containing 10 and 20 ppm monensin and a pH of 6.4 in bottles containing 1, 10 or 20 ppm chloramphenicol.

Table 8.2.11 pH following incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 and 20 ppm of monensin or chloramphenicol.

Control	Monensin (ppm)			Chloramphenicol (ppm)		
(0 ppm)	1	10	20	1	10	20
6.5^{a}	6.5^{a}	6.3^{b}	6.3^{b}	6.4^{c}	6.4^{c}	6.4^{c}

Values not bearing the same superscripts differ significantly ($p < 0.05$), sed = 0.014. The analysis of variance table is shown in Appendix 8.2.10.

8.2.4 Discussion

In order that conditions *in vitro* would be optimal for detecting the effects of antibiotics on the fermentation, the donor animals did not receive any antibiotic supplementation (van Nevel & Demeyer, 1990). In addition, the donor animals were fed a 90 % roughage diet (section 3.2) whilst naked oats were used as the substrate *in vitro*. This enabled the usefulness of the antibiotics at reducing ruminal disorders caused by rapidly increasing the concentrate portion of the diet to be investigated (Beede & Farlin, 1977).

The addition of antibiotics (dissolved in distilled water or a 50:50 distilled water: methanol solution) to the fermentation bottles had a negligible effect on gas production. Occasionally, however, there was a trend for reduced gas production for approximately 20 min after injection of the antibiotic, but this was negligible relative to the total gas production profile. In all instances, gas production quickly recovered and the fermentation proceeded supporting the findings of Beede and Farlin (1977) who reported that the addition of 1 ml aliquots of distilled water or methanol to incubations containing 29 ml of buffered rumen fluid had no effect on rumen fermentation *in vitro*. The small decrease in gas production noted when the antibiotic solutions were added may therefore be a transient response of the system to non-reduced liquids.

Penicillin G was the only antibiotic which, according to parallel curve analysis, had a significant effect, at all concentrations, on both the rate of gas production and the total volume of gas produced during the incubation of naked oats. The volume of gas produced increased with an increase in the concentration of penicillin compared to control bottles with no antibiotic. Incubation with 10 and 20 ppm monensin or 1 ppm chloramphenicol had a similar effect on the gas production profiles as lasalocid and cationomycin supplementation (Bogaert *et al.*, 1989); less gas being produced in the presence of the antibiotics compared to controls. Although, the composition of the gas was not measured, it is likely that the proportion of CH_4 was reduced (Bartley *et al.* 1979; Bogaert *et al.*, 1989). Van Nevel and Demeyer (1992) reported that several antibiotics which increased the molar proportion of propionate also inhibited CH_4 production, whilst those antibiotics which decreased the proportion of propionate were always associated with a significant increase in methanogenesis. These findings are supported by the stoichiometry described by Hungate (1966) and Wolin (1975), whereby the production of acetate and butyrate result in the production of greater volumes of gas than the production of propionate. Incubation with all concentrations of avoparcin, chloramphenicol and 1 ppm monensin did not have a significant effect on the volume of gas produced, thus demonstrating that the activity of the antibiotics is dependent upon concentration. Whilst some antibiotics are more effective at low

concentrations others must be present at higher concentrations in order to demonstrate an effect.

Avoparcin, monensin and chloramphenicol supplementation had no effect on DM loss in this study. This was in contrast to previously reported results where reduced DM loss has been reported with several antibiotics, including monensin (Wallace *et al.*, 1981; Baldwin *et al.*, 1982), lasalocid, cationomycin (Bogaert *et al.*, 1989), virginiamycin, salinomycin and terramycin (van Nevel & Demeyer, 1990). A significant decrease in DM loss did occur during this study however in the presence of penicillin. This decrease may have been a result of penicillin's strong inhibitory action against several micro-organisms, for example, Muir and Barreto (1979) reported that penicillin had the maximum activity against *S. bovis* when compared with several other antibiotics (ampicillin, thiopeptin, tylosin, tetracycline, oxytetracycline, bacitracin, chlortetracycline, gentamicin and streptomycin). It is likely that *S. bovis* will be the main starch degrading organism present during the *in vitro* fermentation and therefore inhibition of this organism would be expected to decrease DM loss from the naked oats.

The effect of the antibiotics on VFA production varied with the different antibiotics. In agreement with Nagaraja *et al.* (1987), avoparcin supplementation increased the proportion of propionate and decreased the proportion of butyrate without affecting the total amount of VFA produced. An increase in the proportion of propionate has also been reported *in vivo*, however the total VFA production decreased (Froetschel *et al.*, 1983). The decrease in total VFA was attributed to an increase in rumen volume (Froetschel *et al.*, 1983). If the decrease in total VFA *in vivo*, is due only to an increase in rumen volume a similar effect will not be observed *in vitro* as fermentations are generally conducted in vessels with a fixed volume.

Supplementation with 20 ppm penicillin G had no effect on total VFA production, however incubation with both 1 and 10 ppm penicillin resulted in a decrease in total VFA, supporting the findings of Beede and Farlin (1977). Unlike avoparcin and

monensin supplementation where propionate production is seen to increase, penicillin supplementation (10 and 20 ppm) led to a decrease in propionate production whilst acetate production increased.

Monensin, at a concentration of 10 ppm, was also seen to increase propionate and decrease butyrate production with no change in total VFA, in agreement with the findings of Bartley *et al.* (1979) and Nagaraja *et al.* (1987). Whilst incubation with 20 ppm increased the total VFA production supporting the findings of Van Nevel and Demeyer (1992), although contradicting those of Beede and Farlin (1977) and Hino *et al.* (1993) where the total VFA production was seen to decrease with the addition of monensin. The effect of monensin on total VFA production is therefore unclear. In all of the above studies the rumen microbial inocula was not adapted to the antibiotics so the conditions in all cases were optimum for the effect of monensin to be observed (van Nevel & Demeyer, 1990). Different substrates were used in the above studies which may explain some of the differences noted, however there is no consistent pattern between similar feedstuffs. For example, the increase in total VFA production noted in this study when naked oats were incubated with 20 ppm monensin was similar to that observed for a poor quality forage (van Nevel & Demeyer, 1992) whilst total VFA production from a more closely related substrate, wheat, was seen to decrease in the presence of monensin (Beede & Farlin, 1977). The effect of monensin on total VFA production is therefore difficult to predict. However, certain aspects of monensin supplementation are well documented, such as the increase in propionate production which occurred in all of the above cases.

Incubation with chloramphenicol had no effect on total VFA production, however at higher concentrations than those used in this study (> 25 ppm), chloramphenicol may reduce total VFA production (Baldwin *et al.*, 1982).

Beede and Farlin (1977) developed the following comparative index in order to describe the influence of antibiotics on apparent lactate and VFA production:

% acid production in antibiotic treated sample =
(Treated sample value - initial 0 h value / control sample value - initial 0 h value) x 100 (equation 8.25)

The relative acetate:propionate (Ac:Pr) ratio can also be compared between the antibiotic treated samples and the control bottles using a similar equation:

Ac:Pr (%) = (Treated sample Ac:Pr / Control sample Ac:Pr) x 100 (equation 8.26)

Therefore the control index value equals 100 with treated sample values being less than or greater than 100. Applying the equation to the data in this study resulted in the following Ac:Pr index values; for avoparcin at 1, 10 or 20 ppm, the index values were 97, 88 and 83 %. The decrease in the index value with the increasing concentration of avoparcin represents a decrease in the Ac:Pr ratio. Incubation with penicillin had the opposite effect, increasing the concentration of penicillin resulting in an increase in the Ac:Pr ratio; with index values of 90, 146 and 219 % for 1, 10 and 20 ppm, respectively. The Ac:Pr index values obtained after incubation with monensin were 93, 81 and 87 % for 1, 10 and 20 ppm respectively. Thus, treated samples had a lower Ac:Pr ratio than controls. Incubation with chloramphenicol resulted in Ac:Pr index values of 96, 94 and 96 % for 1, 10 and 20 ppm, respectively. These values are all close to 100, hence the Ac:Pr ratio is similar to that in control bottles although the index values are all below 100 suggesting that chloramphenicol tends to lower the Ac:Pr ratio.

Accumulation of lactic acid in the rumen can lead to ruminal disorders such as lactic acidosis (section 8.1.2.2.1). Antibiotics which can prevent the accumulation of lactic acid are therefore beneficial to the health of the animal. In this study, total lactate production after incubation with all concentrations of avoparcin, monensin and

chloramphenicol were significantly lower than in control bottles. Nagaraja *et al.* (1987) also reported a significant decrease in lactic acid concentration after 12 h incubation of glucose with both avoparcin and monensin. Penicillin (10 ppm) also significantly reduced the total lactic acid concentration after incubation. However, Beede and Farlin (1977) measured an increase in the lactic acid concentration after incubating wheat in the presence of both monensin and penicillin. This discrepancy may be due to the difference in the time interval used to measure lactic acid production; Beede and Farlin (1977) recorded lactic acid production after 1 h, whilst in this study and the study reported by Nagaraja *et al.* (1987) lactic acid production was recorded after a longer time interval (either 72 or 12 h incubation, respectively). As lactic acid will be used during the incubation, measuring the concentration of lactic acid present earlier in the incubation may be more representative of the activity of the antibiotic. However, if there is less lactic acid present at the end of the incubation in the presence of the antibiotic when compared to controls, the antibiotic must be influencing the fermentation and / or production of lactic acid.

Applying Beede and Farlin's (1977) index to lactic acid concentrations resulted in values of 59, 68 and 41 % for 1, 10 and 20 ppm avoparcin, whilst the values for penicillin were 109, 57 and 85 %, for 1, 10 and 20 ppm respectively. The increase in lactate after incubation with 1 ppm penicillin, although not significant, tends to agree with the findings of Beede and Farlin (1977) where an increase in lactate production was seen. However a significant decrease was noted with 10 ppm penicillin, disagreeing with their findings. Index values for both monensin and chloramphenicol were below 100 indicating a decrease in lactic acid. The values for 1, 10 and 20 ppm monensin were 56, 66 and 61 %, whilst chloramphenicol resulted in index values of 69, 79 and 62 % for 1, 10 and 20 ppm respectively.

The APES appears to be a useful screening technique for investigating the potential of antibiotics as feed supplements; with differences in gas production, DM loss, VFA production and molar proportions and lactic acid production being detected. However, evaluation *in vivo* of those antibiotics which appear to have a beneficial

effect on fermentation is important as *in vitro* techniques will never simulate *in vivo* conditions exactly. For example avoparcin was seen to decrease total VFA production *in vivo* (Froetschel *et al.*, 1983) whilst no effect on total VFA production was seen in this *in vitro* study. *In vitro* techniques may also be unable to detect any toxic effects of the antibiotics, hence the antibiotics should be chosen carefully and used at the lowest effective concentrations. For example, monensin fed at concentrations of 22.4 and 39.8 mg kg^{-1} of body weight caused death in five of ten cattle which received the supplementation (Potter *et al.*, 1984). Therefore certain effects of antibiotic supplementation are unable to be predicted exactly *in vitro*, whilst other aspects of antibiotic supplementation may be predicted fairly accurately.

Chapter 9 - General Discussion

The work of this thesis covered a broad area of research relating to factors which affect the measurement of gas during *in vitro* gas production studies. The results obtained relate the problems associated with the biology and chemistry of gas production to the need to develop rapid *in vitro* procedures for estimating the kinetics of feed degradation. This chapter, firstly, summarises the various factors which influence the measurement of gas in the pressure transducer technique (section 9.1); detailed discussions of the experimental results can be found in the preceding chapters. Secondly, the potential applications of gas production techniques are discussed in section 9.2, and finally, the main conclusions of this thesis are presented in section 9.3.

9.1 Summary of the factors which influence the measurement of gas production

As discussed in chapters 4, 5 and 6, there are several physical, biological and chemical factors which affect the measurement of gas production in the pressure transducer technique. These factors are generally relevant to all techniques which rely on the measurement of gas production and must be taken into consideration when designing experiments and interpreting results from gas production experiments.

9.1.1 Physical factors

The physical factors investigated in this thesis included temperature, head-space pressure and shaking (chapter 4) as well as the physical properties of the substrate; particle size and different drying treatments (chapter 6). Temperature was shown to have a detrimental effect on the fermentation when temperatures were above or below the optimum temperature of 39 °C (section 4.1). Whilst the increase in head-space pressure obtained by decreasing the reading frequency from every 2 h, to every 4 or 6 h did not affect the fermentation (in terms of VFA production, DM loss and change in pH of the culture medium) it had a significant influence on the volume of gas measured (section 4.2). Gas production and reading frequency showed a positive correlation; the lowest volumes of gas were measured when a reading frequency of 6

h was used, more gas being produced when the reading frequency was every 4 h and most when a reading frequency of every 2 h was used. Reducing the quantity of substrate incubated to 0.25 g removed this effect, with no differences in gas production between reading frequencies of 2, 4 or 6 h.

Continual shaking of the culture medium had a similar effect to that of increasing head-space pressure; no differences in VFA production were found between bottles which were shaken continuously after every gas reading or not shaken. However, there were differences in gas production and DM loss. The difference in DM loss may have been due to a physical breakdown of the substrate caused by the shaking movement rather than by an increase in fermentation, since no differences were detected in the quantity or composition of VFA produced. Less gas was measured in continuously shaken bottles compared to those shaken after every gas production reading or those not shaken at all. According to Pell and Schofield (1993) and Cone *et al.* (1996), the main objective of shaking the culture bottles during the fermentation process is to avoid supersaturation which could affect the measurement of gas and the fermentation itself. However, from the results of this study it appears that as long as the gas is removed at frequent intervals throughout the incubation, supersaturation does not occur.

The form of the substrate, in terms of particle size and drying treatment, also influenced the gas production profile. In general, a decrease in particle size tended to increase gas production by increasing the surface area available to the micro-organisms for attachment. However there was little difference between gas production profiles for different particle sizes of naked oats (mean geometric diameter ranging from 6.022 mm to < 1 mm). In contrast the gas production profiles for various hay particle sizes (mean geometric diameter ranging from 3.098 mm to 0.212 mm) showed distinct differences in their gas production profiles. The smallest particles produced most gas; less gas was produced as particle size increased (chapter 6). The methods used in feed evaluation techniques must be rapid and accurate, hence, although a large proportion of the particles ingested by an animal (especially

ruminants) may be greater than 1 mm, the results of this experiment suggest that incubation of small particles (< 1 mm), which are degraded quickly and produce less variable results than large particle sizes, are favoured (Johnson, 1966; Nocek, 1988; Weiss, 1994). The particle size of the feed will also change with time after ingestion and hence its rate of digestion will also change with time. Thus it is important to be aware of the relationship between particle size and the volume of gas produced in these *in vitro* procedures to ensure correct interpretation of the results obtained. The majority of *in vitro* digestibility techniques require that samples are dried prior to evaluation, enabling the samples to be stored, ground and sampled homogeneously. However, the experiment detailed in chapter 6, showed significant differences between the gas production profiles obtained during the incubation of oven, microwave or freeze dried samples of ryegrass when compared to fresh ryegrass. There were also differences in the gas production profiles obtained between the various drying treatments, highlighting the importance of incubating feeds treated in the way in which they are to be fed, in order to accurately predict what will happen to these feeds *in vivo*.

The results of these studies indicate that all these factors must be considered when developing experiments and interpreting gas production data. Incubation temperature can be easily controlled by the use of an incubator, water bath or constant temperature room. Head-space pressure will be an important consideration in studies where botanically diverse feedstuffs are to be compared (as there will be a wide range in the rate and volume of gas produced) but will be less critical when similar feedstuffs are compared (as the rate and quantity of gas production are likely to be similar). Increases in head-space pressure are difficult to overcome with the manual pressure transducer technique; the pressure in the head-space would have to be monitored almost continuously in order to determine when to release the gas. However, automated gas production techniques, in which the culture bottles are vented when the gas pressure in the head-space has reached a preset value (for example, Davies *et al.*, 1995; Cone *et al.*, 1996) eliminate such problems. For routine feed analysis particle size should be standardised to a relatively small size (< 1 mm)

in order to minimise incubation times and to reduce variation in experimental results. However for research purposes such as investigating the potential benefit of feed additives for increasing digestibility (for example the addition of enzymes or ammonium hydroxide to cereal grains, straws or silages) differences may only be detected if the samples are incubated in a similar form to which they would be fed. For example, the gas production profiles of enzyme-treated silages were the same as for untreated samples when all samples had been ground before incubation. However, when samples of the treated and untreated silages were incubated in their original short chop form, differences in gas production were measured (Z. Davies and M.K. Theodorou, IGER, Aberystwyth - personal communication). Differences in gas production profiles are also seen when samples are dried, therefore, ideally the feedstuff should be analysed in the form it is to be fed. If this is not possible freeze drying, oven drying (60 °C) and microwave drying may all provide suitable alternatives, but caution should be used in extrapolating from the data to the animal.

9.1.2 Biological and chemical factors

The biological and chemical factors investigated were the nature of the feedstuff (fermentation pattern), the type of buffer and the effect of different sources of inoculum.

The nature of a feedstuff has a significant effect on gas production profiles. Feeds rich in starch produced molar VFA percentages of the order 46 Ac: 43 Pr: 9 Bu: 2 Val (naked oats; section 5.1) whilst fibre based feeds produced molar VFA percentages of the order 74 Ac: 17 Pr: 7 Bu: 2 Val (oatfeed; section 5.1). These profiles are fairly similar to those measured in the rumen of animals fed similar feedstuffs (McDonald *et al.*, 1995; Rymer & Givens, 1998). The volume of gas produced from the formation of individual VFA differs (Hungate, 1966; chapter 5), and there is therefore a need to standardise gas production profiles to a given VFA molar ratio. Problems will arise in this context when gas production is used to rank a diverse group of feeds in terms of their digestibility. Low digestibility feeds with high acetate values may rank above higher digestibility feeds with lower acetate and

higher propionate values. Similarly, lag time at the start of the incubation is affected by the VFA produced; a long lag time may indicate the production of propionate rather than minimal fermentation. Hence, although gas production techniques may be useful for ranking similar feedstuffs, care should be taken when comparisons are made between widely different feeds. One solution to this problem would be to correct all gas production profiles to a standard VFA profile (chapter 5).

The interpretation of gas production profiles is complicated further by the neutralisation of VFA by bicarbonate buffer present in the culture medium. Chapter 5 details an experiment where acidification gas was measured by stepwise addition of individual VFA to the culture medium. This gave a value for the conversion of each mmol VFA produced to ml acidification gas formed (all values were < 1 as the medium contained both a phosphate and a bicarbonate buffer). These values together with the stoichiometry described by Hungate (1966) were used in an attempt to predict gas production. However, the predicted gas production did not correspond with the measured values. Mauricio *et al.* (1998) have demonstrated that both the quantity of VFA produced and the rate at which they are produced affects the rate and total volume of acidification gas produced hence, the estimation of acidification gas may not have been appropriate in this experimental situation. This problem could be overcome by using a phosphate only buffer in the culture medium however, at present, bicarbonate ions are necessary in order to sustain the micro-organisms (Pell & Schofield, 1993). It is clear that more research on the production of acidification gas is required and that alternatives to the bicarbonate buffer should be sought. Other possible explanations to explain the discrepancy between the predicted and experimental gas production include loss of VFA from the culture medium into the head-space, the production of gases other than those produced by the formation of VFA, for example, hydrogen sulphide, ammonia and water vapour and the stoichiometry used may have to be adapted to account for the presence of pentose sugars as well as hexose sugars (section 5.1.4).

As expected, gas production profiles obtained using different sources of microbial inocula varied between sources. During the incubation of fibrous feedstuffs, oatfeed, soya hulls and unmolassed sugar beet pulp, gas production was greatest with rumen fluid inoculum, whilst equine caecal and faecal inocula produced similar gas production profiles. For naked oats, most gas was produced during incubation with the equine caecal and faecal inocula; least was produced during incubation with the rumen fluid inoculum. The similarity between gas production profiles produced during incubation with the equine caecal and faecal inocula suggest that equine faeces are a suitable alternative to caecal digesta as a source of inocula for *in vitro* gas production studies. These findings were supported by the results of the experiment detailed in section 8.1, where the prediction equations developed from gas production data showed good correlations with the actual *in vivo* data for the digestibility of equine feeds.

9.2 Applications for gas production techniques

Pressure transducers used in gas production studies detect pressure through a diaphragm element, this force is then converted into an electrical signal which, in the case of the pressure transducer technique of Theodorou *et al.* (1994) is displayed as a digital output (i.e. the pressure is converted to a numerical value directly equivalent to its written value) (McGraw-Hill Encyclopaedia of Science and Technology, 1997). The first pressure transducer technique for measuring gas production from micro-organisms was developed in 1974 as a quality control tool (Wilkins, 1974). The technique consisted of a 5.0 psi pressure transducer and a pressure equaliser valve attached to the metal cap of a test tube containing cultures of either *Escherichia coli, Enterobacter aerogenes* or *Citrobacter intermedium*; the gas pressure in the test tube was recorded on a strip chart recorder over a 24 h incubation period. Suggested applications for the technique were the detection of coliforms in water supplies and the presence of pathogenic micro-organisms in clinical preparations or food samples. Gas production techniques are still used as research tools for investigating the growth of various micro-organisms (Theodorou *et al.*, 1995 & 1996a,b). Their main advantage is that unlike gravimetric techniques, where several culture bottles are

required to obtain growth curves for individual micro-organisms, an entire growth curve can be obtained from a single culture bottle (Theodorou *et al.*, 1995). In addition, Theodorou *et al.* (1995) found that for anaerobic fungi growing on glucose a 1:1 ratio was obtained between specific growth rates as determined by dry weight and gas production measurements. Gas production techniques are also becoming increasingly popular for estimating the apparent digestibility and fermentation kinetics of animal feeds. For example, gas production parameters have been correlated with several *in vivo* parameters, such as digestibility (Khazaal *et al.*, 1993), feed intake and growth rate (Blummel & Orskov, 1993) as well as with *in situ* degradability (Sileshi *et al.*, 1996).

In chapter 8, gas production measurements were found to correlate well with both DMIV and DE suggesting the use of the gas production technique as a routine feed evaluation tool. However, the results in this thesis suggest that in order for gas production techniques to provide a repeatable, accurate technique for feed evaluation, the following criteria must be met:

1. Only one gas production technique should be used.

The comparison of the pressure transducer technique (Theodorou *et al.*, 1994) with the Menke *et al.* (1979) technique (chapter 7) illustrated significant differences between the resulting gas production profiles, therefore for routine feed analysis the same gas production technique should be used in all laboratories. The automated gas production techniques [for example, Pell & Schofield (1993), Davies *et al.* (1995) and Cone *et al.* (1996)] would be more suited to this purpose than the manual techniques [for example, Menke *et al.* (1979) and Theodorou *et al.* (1994)] as the automated recording of gas production does not need to be supervised at frequent intervals throughout the incubation. In addition the techniques of Davies *et al.* (1995) and Cone *et al.* (1996) may be preferred to the technique described by Pell & Schofield (1993) as the gas is vented when the pressure reaches a certain level in the head-space, thus avoiding problems caused by an increase in head-space pressure.

2. Source, collection and preparation of microbial inoculum must be standardised. In a similar way to the Tilley and Terry (1963) technique, the microbial inoculum needs to be standardised. Firstly the type of inocula should be standardised, that is whether the inocula source is faeces or rumen fluid. At present rumen fluid is the most common source of microbial inoculum for feed evaluation techniques. Faeces however does not require the use of surgically prepared animals and can therefore be collected from a greater number of animals. A suggested protocol for the source, collection and preparation of microbial inoculum for analysing forages could be as follows: freshly voided faeces should be collected (within 1 h of voiding) between 8.00 and 10.00 am, from four cattle fed hay *ad libitum*. Faeces should be blended in a liquidiser (to obtain particle associated micro-organisms) then strained twice through three layers of muslin. Throughout the process the inoculum should be flushed with CO_2 gas and gently mixed with a magnetic stirrer and bar. For the analysis of concentrate feeds, collection and preparation of the microbial inoculum should follow the above protocol, however the source of inoculum should be faeces from four cattle fed a standard 50:50 concentrate:forage diet.

3. The culture medium used by all laboratories should be standardised. Modified Van Soest medium (as described in chapter 3) appeared to be suitable for incubation of both forage and concentrate feeds. However further research into the production of acidification gas from the bicarbonate - phosphate buffer, or alternative buffer sources which would avoid the production of acidification gas is required.

4. The medium : microbial inoculum ratio should be standardised. A ratio of 9:1 has been used successfully throughout this thesis and is therefore suggested as a suitable standard for routine feed analysis. However, further research is needed to ensure that this is the correct concentration to use.

5. The weight of feedstuff incubated and preparation of samples for incubation should also be standardised. For example the quantity of feedstuff incubated should

be 1.00 g DM and all feeds should be freeze dried, then ground through a 1 mm dry mesh screen before incubation.

6. Gas production profiles should be corrected to a standard VFA ratio.

As discussed earlier, the formation of different VFA results in the production of different quantities of gas and it is therefore important to standardise gas production profiles to a common VFA profile. However, this would increase the complexity of the technique making it less suitable for routine feed analysis, although the problem may be overcome by developing individual prediction equations for different groups of similar feedstuffs.

7. Each laboratory must develop its own prediction equations.

As discussed in section 2.1.4 it will be important for each laboratory to develop its own prediction equations for nutritive values using gas production measurements. This should be done using gas production measurements, obtained in that laboratory, for a standard group of test feeds with known *in vivo* nutritive values.

8. Modelling procedures and statistical analysis should also be standardised.

At present there are several models available for describing gas production profiles (section 2.3). In order to compare gas production parameters between laboratories it will be important to standardise the model which is used to describe gas production profiles. Qualities required by the model will include the ability to fit a wide range of gas production profiles as well as being 'user friendly' in order that it can be adopted universally. In addition to a standard model, a standard method of statistical analysis must also be adopted. In this thesis parallel curve analysis and analysis of variance have both been used to compare gas production profiles. Parallel curve analysis compared the mean gas production profiles for each treatment whilst analysis of variance utilised data from the individual replicates within each treatment. As the replication used in the analysis of variance was within experiment replication, significant differences were detected between treatments even when the differences were relatively small. However, parallel curve analysis which was used to compare

the mean gas production profiles for each treatment also detected significant differences between most treatments.

Due to limitations in resources, proper experimental replication (several experimental runs or collecting digesta / faeces from three different animals and using these to prepare three separate inoculum sources) was not carried out in this thesis. The replication therefore accounted for laboratory error rather than experimental error and small differences appeared significant when in fact they may not have been biologically different. Although this is an important consideration many methods for feed evaluation do not account for experimental error as increasing the number of experimental runs or using three separate inoculum sources greatly increases the time required for feed evaluation.

Gas production for research

Although gas production data have been successfully used to predict several *in vivo* parameters (section 8.1; Blummel & Ørskov, 1993; Khazaal *et al.*, 1993) for routine feed evaluation NIR techniques may be preferable to gas production techniques. NIR analysis requires minimal labour input whilst producing maximal data output and is therefore best suited to routine feed evaluation. However, the initial cost of NIR equipment is generally greater than that of gas production techniques and gas production techniques may be used to validate and up-date NIR calibration sets (section 2.1.5).

For research purposes the criteria for gas production studies are less strict and the particular aim of the research will decide the protocol for the gas production study. Examples of possible research applications for gas production techniques and the most important criteria in terms of experimental protocol associated with that aim are summarised in Table 9.1.

Table 9.1 Application of the gas production technique as a research tool.

Research interest	Experimental criteria
Feed additives	samples should be incubated in the form in which they would be fed as opposed to being ground through a 1 mm mesh screen
Composition of gas produced (for example methane production)	techniques where the gas produced is not vented automatically are required or techniques could be adapted so that gas vents automatically into a gas analyser
Protein degradation	the culture medium should not provide a nitrogen source
Microbial growth curves	requires pure (ascenic) cultures

Gas production techniques have been used for the evaluation of feeds for cattle, sheep and pigs. Several other *in vitro* techniques have been developed for predicting *in vivo* digestibility in these animals however, information on the digestibility of different feeds for equines is scarce. Reliable methods of feed evaluation are essential for formulating diets so that they meet the requirements of the horse for production and performance (Harris, 1997; Frape, 1998). The experiment detailed in section 8.1 showed that the use of equine faeces as a source of inoculum in the gas production technique may be a suitable method for predicting the digestibility of feeds for equines. The use of faeces as an inoculum greatly increases the application of the technique, providing a cheap, readily available, microbial inoculum. It also provides an opportunity for investigating fermentation in animals with compromised gut function. For example, faecal samples could be collected from an animal showing signs of a compromised gut function (for example, diarrhoea) and used within the gas production technique. The resulting gas production profiles could be compared with those obtained from the same feed during incubation with healthy equine faeces as

the inoculum source. Different drugs could then be administered *in vitro* to determine those which may be beneficial in the treatment of the condition. Alternatively, the technique could be used to assay the effect of oral antibiotics on hindgut function.

The use of faeces as an inoculum source for *in vitro* incubations has greatly increased the potential and accessibility of gas production techniques for feed evaluation. Gas production techniques have been used to investigate the gas producing properties of baby foods using human faeces as the inocula source (Cone *et al.*, 1997). This approach has enabled the exclusion of foods that produce large quantities of gas from the final product in order to avoid large volumes of gas accumulating in the colon. Gas production techniques can also be applied to investigate the effect of enzymes, chemicals or genetic modifications on the digestibility of plants or other substrates (Beuvink, 1993). Similarly, they can also be useful as screening tools for investigating which potentially beneficial feed additives should be investigated *in vivo* (for example antibiotics; chapter 8). The automated pressure evaluation system (APES) used in chapter 8, provided a quick, easy method for screening antibiotics and was able to detect small differences between treatments. Hence, this technique may be applied as an initial screening tool for novel feed additives such as probiotics and natural plant extracts.

Gas production techniques are preferable to *in vivo* digestibility trials in terms of the requirement for a number of animals, cost, time and the labour involved. Gas production techniques also have several advantages over *in situ* techniques; fewer animals are required for *in vitro* gas production studies compared to *in situ* studies and fistulated animals are not required (faeces being a suitable alternative inocula source). In addition they can provide more information about the feedstuff in terms of the contribution of the soluble fraction to the fermentation (*in situ* soluble material is lost from the bags and is therefore considered to be instantaneously degraded, whilst *in vitro* gas production from the soluble fraction can be measured and its degradation kinetics determined: chapter 5).

9.3 Conclusions

The pressure transducer technique and gas production techniques in general have several applications as research tools for anaerobic microbiology and animal nutrition studies. They are generally simple to use and provide accurate, reproducible results. However, caution must be exercised when interpreting gas production data. Many factors affect the measurement of gas production and uncertainty remains about the origin of the gas which is measured. For this reason the technique may, at present, be more suited to general research as opposed to a routine method of feed evaluation, and further research is required before the technique can be routinely used as a method of feed evaluation. Further research should also include analysis of the gas which is produced during fermentation to gain a better understanding of the processes which are occurring. This could also be used to help reduce the production of greenhouse gases by determining diets and examining the potential of various feed supplements for their ability to reduce the quantity of methane produced during rumen fermentation (Moss, 1994; McAllister *et al.*, 1996).

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APPENDIX 1

The chemical composition of the feedstuffs used throughout this thesis.

Sample	DM	(g kg^{-1} DM)			
	(g kg^{-1})	ADF	NDF	OM	CP
Hay[1]	938.5	347.8	648.6	953.6	61.3
Naked oats	901.1	26.5	131.9	984.7	180.1
Oatfeed	893.2	435.2	852.3	965.3	21.5
Soya hulls	894.6	457.3	635.7	949.8	120.8
Sugar beet	907.8	265.6	527.9	847.6	76.5
Hay[2]	936.2	384.1	669.7	960.7	68.7
Ryegrass[3]	185.1	226.1	430.4	927.1	109.2
White clover	198.0	181.3	222.0	866.3	297.2
Dried Ryegrass[4]	921.0	-	362.0	926.5	158.0

[1]Grass hay (*Lolium perenne*) fed to both fistulated ponies and steers.

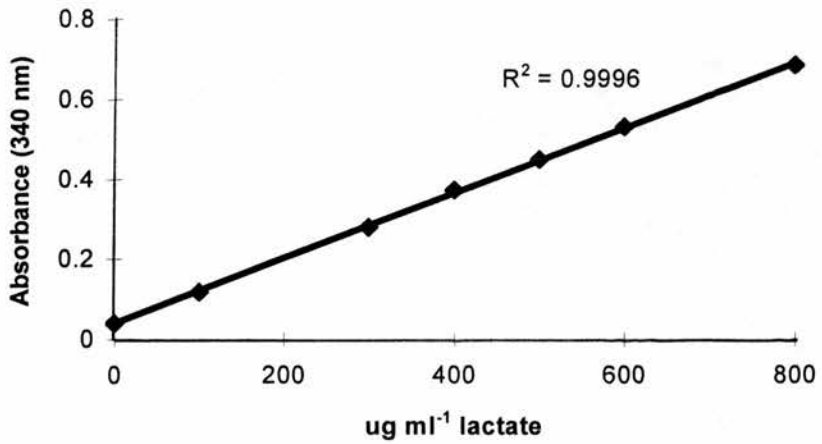
[2]Grass hay (*Lolium perenne*) used in shaking, head-space pressure, temperature experiments (chapter 4)

[3]Ryegrass used in chapter 5 - biological factors affecting gas production; clover versus grass mixtures.

[4] Dried Ryegrass (*Lolium multiflorum*) used in chapter 7 - comparison of the PTT and MT.

APPENDIX 2

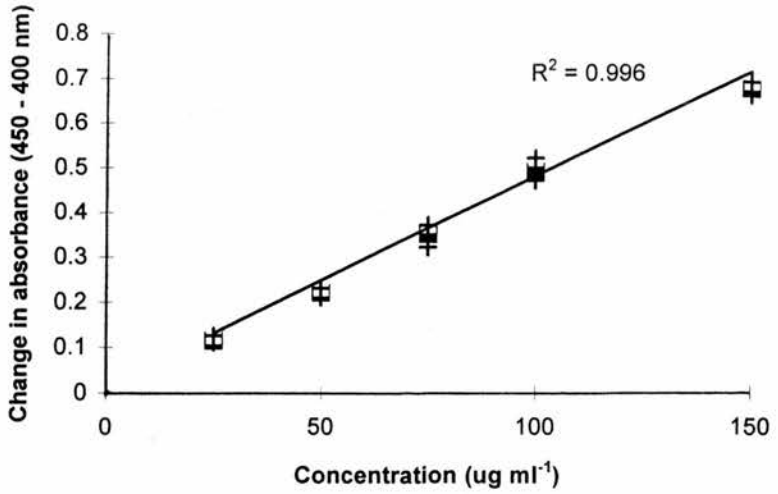
Standard curve for the determination of total lactic acid (μg ml^{-1})



The absorbance of different concentrations of 50:50 mixtures of D- and L- lactic acid were determined as described in chapter 3. The absorbance of the lactic acid mixtures was read at 340 nm.

APPENDIX 3

Calibration curve for the determination of uronic acid concentration in feed samples



Uronic acid concentration was determined as described in section 3.12. The above standard calibration curve was developed to determine the concentration of uronic acid present in test samples. The standards consisted of glucoronic acid at the following concentrations: 25, 50, 75, 100 and 150 μg ml^{-1}. The mean absorbance values from two sets of standards were determined. The absorbance measured at a wavelength of 400 nm was subtracted from values for absorbance measured at 450 nm and the average values plotted against uronic acid concentration to obtain the standard curve. The R^2 value for the above curve was 0.996, where + represents the actual values from two standards and ■ represents the mean value of the standards.

APPENDIX 4

Appendix 4.1.1 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 0.75 g hay incubated with a rumen microbial inoculum at 25, 30, 39 or 45 °C.

Temperature	Source[1]	DF	SS	MS	F-ratio[2]
25 vs 30 °C	Between rates (b and c)	2	160.9604	80.4802	27.4836***
	Between gas pool (A and B)	2	23107.6900	11553.8500	3945.5800***
	within subset	22	64.4220	2.9283	
25 vs 39 °C	Between rates (b and c)	2	76.2976	38.1488	11.3972***
	Between gas pool (A and B)	2	115176.7000	57588.3600	17204.94***
	within subset	22	73.6386	3.3472	
25 vs 45 °C	Between rates (b and c)	2	31.1955	15.5977	17.0616***
	Between gas pool (A and B)	2	68860.5200	3443.2600	37661.6300***
	within subset	22	20.1124	0.9142	
30 vs 39 °C	Between rates (b and c)	2	2739.8420	1369.9210	224.1363***
	Between gas pool (A and B)	2	36820.8600	18410.4300	3012.1777***
	within subset	22	134.4635	6.1120	
30 vs 45 °C	Between rates (b and c)	2	1700.9090	850.4544	231.1646***
	Between gas pool (A and B)	2	13711.5600	6855.7790	1863.4898***
	within subset	22	80.9372	3.6790	
39 vs 45 °C	Between rates (b and c)	2	88.6351	44.3176	10.7651***
	Between gas pool (A and B)	2	5957.8770	2978.9390	723.6055***
	within subset	22	90.5699	4.1168	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

***$p < 0.001$, the 0.1% value for F was 9.612 (Lindley & Scott, 1990).

Appendix 4.1.2 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of perennial ryegrass hay (*Lolium perenne*) with rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	0.0066	0.0022	821.72	< 0.001
Replicate	2	0.0000011	0.0000005	0.20	0.824
Residual	6	0.0000161	0.0000027		
Total	11	0.00662			

Appendix 4.1.3 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of perennial ryegrass hay (*Lolium perenne*) with rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	0.153744	0.051248	1369.47	< 0.001
Replicate	2	0.000098	0.000049	1.31	0.337
Residual	6	0.0002245	0.000037		
Total	11	0.1540667			

Appendix 4.1.4 The cumulative gas production, A (ml; France *et al.*, 1993) obtained during incubation of perennial ryegrass hay (*Lolium perenne*) with rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	47427.11	15809.04	240.94	< 0.001
Replicate	2	207.79	103.90	1.58	0.280
Residual	6	393.69	65.61		
Total	11	48028.59			

Appendix 4.1.5 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of perennial ryegrass hay (*Lolium perenne*) with rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	31047.37	10349.12	176.72	< 0.001
Replicate	2	149.18	74.59	1.27	0.346
Residual	6	351.37	58.56		
Total	11	31547.92			

Appendix 4.1.6 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of perennial ryegrass hay (*Lolium perenne*) with rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	2.1499	0.7166	205.83	< 0.001
Replicate	2	0.0263	0.0131	3.78	0.087
Residual	6	0.0209	0.0035		
Total	11	2.1971			

Appendix 4.1.7 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of perennial ryegrass hay (*Lolium perenne*) with rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	177.554	59.185	20.70	0.001
Replicate	2	0.637	0.319	0.11	0.896
Residual	6	17.157	2.859		
Total	11	195.348			

Appendix 4.1.8 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of perennial ryegrass hay (*Lolium perenne*) with rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	23040.9	7680.3	33.82	< 0.001
Replicate	2	148.2	74.1	0.33	0.734
Residual	6	1362.5	227.1		
Total	11	24551.6			

Appendix 4.1.9 Dry matter loss from perennial ryegrass hay (*Lolium perenne*) during 144 h incubation with rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	1008843	336281	180.31	< 0.001
Time	5	878009	175602	94.16	< 0.001
Temperature .Time	15	315307	21020	11.27	< 0.001
Replicate	2	6908	3454	1.85	0.168
Residual	46	85790	1865		
Total	71	2294857			

Appendix 4.1.10 Acetate production (molar %) from perennial ryegrass hay (*Lolium perenne*) during 144 h incubation with a rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	1284.320	428.107	159.48	< 0.001
Time	5	393.775	78.755	29.34	< 0.001
Temperature .Time	15	98.758	6.584	2.45	0.010
Replicate	2	1.130	0.565	0.21	0.811
Residual	46	123.484	2.684		
Total	71	1901.466			

Appendix 4.1.11 Propionate production (molar %) from perennial ryegrass hay (*Lolium perenne*) during 144 h incubation with a rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	1172.557	390.852	148.68	< 0.001
Time	5	509.450	101.890	38.76	< 0.001
Temperature .Time	15	96.024	6.402	2.44	0.011
Replicate	2	2.833	1.417	0.54	0.587
Residual	46	120.923	2.629		
Total	71	1901.787			

Appendix 4.1.12 Butyrate production (molar %) from perennial ryegrass hay (*Lolium perenne*) during 144 h incubation with a rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	17.9146	5.9715	13.92	< 0.001
Time	5	34.9977	6.9995	16.32	< 0.001
Temperature .Time	15	17.5823	1.1722	2.73	0.005
Replicate	2	0.0365	0.0183	0.04	0.958
Residual	46	19.7320	0.4290		
Total	71	90.2631			

Appendix 4.1.13 Valerate production (molar %) from perennial ryegrass hay (*Lolium perenne*) during 144 h incubation with a rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	32.9964	10.9988	57.30	< 0.001
Time	5	7.5875	1.5175	7.91	< 0.001
Temperature .Time	15	9.4833	0.6322	3.29	< 0.001
Replicate	2	0.2733	0.1367	0.71	0.496
Residual	46	8.8295	0.1919		
Total	71	59.1700			

Appendix 4.1.14 Total volatile fatty acid (VFA) production (mmol l^{-1}) from perennial ryegrass hay (*Lolium perenne*) during 144 h incubation with a rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Temperature	3	8539.839	2846.613	568.09	< 0.001
Time	5	10859.538	2171.908	433.44	< 0.001
Temperature .Time	15	1597.703	106.514	21.26	< 0.001
Replicate	2	1.193	0.596	0.12	0.888
Residual	46	230.498	5.011		
Total	71	21228.769			

Appendix 4.1.15 pH during 144 h incubation of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum at 25, 30, 39 or 45 °C.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Temperature	3	0.225743	0.075248	59.35	< 0.001
Time	5	0.577611	0.115522	91.12	< 0.001
Temperature .Time	15	0.211081	0.014072	11.10	< 0.001
Replicate	2	0.007332	0.003666	2.89	0.068
Residual	36 (10)	0.045640	0.001268		
Total	61 (10)	0.830769			

Appendix 4.2.1.1 Parallel curve analysis of the fitted parameters of gas production (France et al., 1993) from 1.00 g naked oats incubated with a rumen microbial inoculum using the pressure transducer technique with gas production recorded every 2, 4 or 6 h.

Reading interval	Source[1]	DF	SS	MS	F-ratio[2]
2 h vs 4 h	Between rates (b and c)	2	675.0955	337.5477	8.2428**
	Between gas pool (A and B)	2	1827.6440	913.8221	22.315***
	within subset	28	1146.6200	40.9507	
2 h vs 6 h	Between rates (b and c)	2	941.5819	470.7910	10.4267***
	Between gas pool (A and B)	2	7906.8580	3953.4290	87.5573***
	within subset	24	1083.6610	45.1525	
4 h vs 6 h	Between rates (b and c)	2	202.1125	101.0563	13.6341***
	Between gas pool (A and B)	2	2095.4500	1047.7250	141.3552***
	within subset	12	88.9440	7.4120	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

**p < 0.01, ***p < 0.001. The F value at the 1 % level was 5.453, 5.614 and 6.927 for 28, 24 and 12 degrees of freedom (df) respectively. The 0.1% value for F was 8.931, 9.339 and 12.97 for 28, 24 and 12 df respectively. (Lindley & Scott, 1990).

Appendix 4.2.1.2 Parallel curve analysis of the fitted parameters of gas production (France et al., 1993) from 0.75 g hay incubated with a rumen microbial inoculum using the pressure transducer technique with gas production recorded every 2, 4 or 6 h.

Reading interval	Source[1]	DF	SS	MS	F-ratio[2]
2 h vs 4 h	Between rates (b and c)	2	75.6713	37.8357	21.6130***
	Between gas pool (A and B)	2	874.7864	437.3932	249.8533***
	within subset	22	38.5126	1.7506	
2 h vs 6 h	Between rates (b and c)	2	94.4029	47.2014	25.5626***
	Between gas pool (A and B)	2	897.5132	448.7566	243.0309***
	within subset	19	35.0835	1.8465	
4 h vs 6 h	Between rates (b and c)	2	6.1872	3.0936	5.5610*
	Between gas pool (A and B)	2	64.7971	32.3986	58.2394***
	within subset	9	5.0063	0.5563	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The F value at the 5 % level was 3.443, 3.522, 4.256 for 22, 19 and 9 degrees of freedom (df) respectively. The 1% value for F was 5.719, 5.926 and 8.022 for 22, 19 and 9 df respectively. Whilst the F value at the 0.1 % was 9.612, 10.16 and 16.39 for 22, 19 and 9 df respectively (Lindley & Scott, 1990).

Appendix 4.2.1.3 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of 1.00 g naked oats incubated with a rumen microbial inoculum using the pressure transducer technique with gas production recorded every 2, 4 or 6 h.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	0.041115	0.020558	286.32	< 0.001
Replicate	2	0.000017	0.000009	0.12	0.890
Residual	4	0.000287	0.000072		
Total	8	0.041420			

Appendix 4.2.1.4 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of 1.00 g naked oats incubated with a rumen microbial inoculum using the pressure transducer technique with gas production recorded every 2, 4 or 6 h.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	0.95155	0.47577	205.02	< 0.001
Replicate	2	0.00406	0.00203	0.88	0.484
Residual	4	0.00928	0.00232		
Total	8	0.96489			

Appendix 4.2.1.5 The cumulative gas production, A (ml; France *et al.*, 1993) obtained during incubation of 1.00 g naked oats incubated with a rumen microbial inoculum using the pressure transducer technique with gas production recorded every 2, 4 or 6 h.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	700.23	350.12	27.96	0.004
Replicate	2	16.69	8.35	0.67	0.563
Residual	4	50.08	12.52		
Total	8	767.01			

Appendix 4.2.1.6 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of 1.00 g naked oats incubated with a rumen microbial inoculum using the pressure transducer technique with gas production recorded every 2, 4 or 6 h.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	23286.7	11643.4	60.77	0.001
Replicate	2	398.3	199.1	1.04	0.433
Residual	4	766.4	191.6		
Total	8	24451.4			

Appendix 4.2.1.7 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of 1.00 g naked oats incubated with a rumen microbial inoculum using the pressure transducer technique with gas production recorded every 2, 4 or 6 h.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	6.65055	3.32528	50.59	0.001
Replicate	2	0.20459	0.10229	1.56	0.316
Residual	4	0.26293	0.06573		
Total	8	7.11807			

Appendix 4.2.1.8 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of 1.00 g naked oats incubated with a rumen microbial inoculum using the pressure transducer technique with gas production recorded every 2, 4 or 6 h.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	5.68156	2.84078	68.22	< 0.001
Replicate	2	0.18825	0.09412	2.26	0.220
Residual	4	0.16656	0.04164		
Total	8	6.03637			

Appendix 4.2.1.9 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of 1.00 g naked oats incubated with a rumen microbial inoculum using the pressure transducer technique with gas production recorded every 2, 4 or 6 h.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	155.0593	77.5296	501.52	< 0.001
Replicate	2	0.1860	0.0930	0.60	0.591
Residual	4	0.6184	0.1546		
Total	8	155.8636			

Appendix 4.2.1.10 The effect of reading bottles every 2, 4 or 6 h on the resulting dry matter loss from 1.00 g naked oats incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	1902.5	951.3	4.75	0.020
Incubation time	3	57494.2	19164.7	95.76	< 0.001
Reading interval.Incubation time	6	2476.5	412.7	2.06	0.102
Replicates	2	572.9	286.5	1.43	0.261
Residual	21 (1)	4202.9	200.1		
Total	34 (1)	59716.2			

Appendix 4.2.1.11 The effect of reading bottles every 2, 4 or 6 h on the resulting dry matter loss from 0.75 g hay incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	2375.1	1187.5	4.25	0.027
Incubation time	3	467103.0	155701.0	557.29	< 0.001
Reading interval.Incubation time	6	4424.8	737.5	2.64	0.044
Replicates	2	261.9	131.0	0.47	0.632
Residual	22	6146.5	279.4		
Total	35	480311.3			

Appendix 4.2.1.12 The effect of reading bottles every 2, 4 or 6 h on the total volatile fatty acid production (mmol l^{-1}) from 1.00 g naked oats incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	62.74	31.37	0.80	0.460
Incubation time	3	3893.97	1297.99	33.25	< 0.001
Reading interval.Incubation time	6	195.81	32.63	0.84	0.555
Replicates	2	132.67	66.34	1.70	0.206
Residual	22	858.77	39.03		
Total	35	5143.96			

Appendix 4.2.1.13 The effect of reading bottles every 2, 4 or 6 h on the acetate production (molar %) from 1.00 g naked oats incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	0.3882	0.1941	0.84	0.443
Incubation time	3	5.0788	1.6929	7.36	0.001
Reading interval.Incubation time	6	2.6052	0.4342	1.89	0.128
Replicates	2	0.1724	0.0862	0.38	0.692
Residual	22	5.0576	0.2299		
Total	35	13.3022			

Appendix 4.2.1.14 The effect of reading bottles every 2, 4 or 6 h on the propionate production (molar %) from 1.00 g naked oats incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	3.8470	1.9235	2.54	0.102
Incubation time	3	144.8656	48.2885	63.65	< 0.001
Reading interval.Incubation time	6	5.2492	0.8749	1.15	0.366
Replicates	2	2.0658	1.0329	1.36	0.277
Residual	22	16.6910	0.7587		
Total	35	172.7185			

Appendix 4.2.1.15 The effect of reading bottles every 2, 4 or 6 h on the butyrate production (molar %) from 1.00 g naked oats incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	3.3816	1.6908	3.56	0.046
Incubation time	3	100.5088	33.5029	70.53	< 0.001
Reading interval.Incubation time	6	2.1887	0.3648	0.77	0.603
Replicates	2	1.6380	0.8190	1.72	0.202
Residual	22	10.4511	0.4750		
Total	35	118.1683			

Appendix 4.2.1.16 The effect of reading bottles every 2, 4 or 6 h on the valerate production (molar %) from 1.00 g naked oats incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	0.02109	0.01054	0.41	0.671
Incubation time	3	16.61070	5.53690	213.61	< 0.001
Reading interval.Incubation time	6	0.10131	0.01689	0.65	0.689
Replicates	2	0.00807	0.00404	0.16	0.857
Residual	22	0.57026	0.02592		
Total	35	17.31143			

Appendix 4.2.1.17 The effect of reading bottles every 2, 4 or 6 h on the total volatile fatty acid production (mmol l^{-1}) from perennial ryegrass hay incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	23.74	11.87	0.90	0.423
Incubation time	3	3934.80	1311.60	98.94	< 0.001
Reading interval.Incubation time	6	44.40	7.40	0.56	0.758
Replicates	2	10.01	5.01	0.38	0.690
Residual	22	278.40	13.26		
Total	35	4032.71			

Appendix 4.2.1.18 The effect of reading bottles every 2, 4 or 6 h on the acetate production (molar %) from perennial ryegrass hay incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	9.0632	4.5316	5.18	0.014
Incubation time	3	39.4044	13.1348	15.00	< 0.001
Reading interval.Incubation time	6	5.4331	0.9055	1.03	0.430
Replicates	2	0.5270	0.2635	0.30	0.743
Residual	22	19.2594	0.8754		
Total	35	73.6871			

Appendix 4.2.1.19 The effect of reading bottles every 2, 4 or 6 h on the propionate production (molar %) from perennial ryegrass hay incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	1.4541	0.7271	1.39	0.271
Incubation time	3	142.7617	47.5872	90.67	< 0.001
Reading interval.Incubation time	6	4.3498	0.7250	1.38	0.266
Replicates	2	0.2633	0.1317	0.25	0.780
Residual	22	11.5459	0.5248		
Total	35	160.3749			

Appendix 4.2.1.20 The effect of reading bottles every 2, 4 or 6 h on the butyrate production (molar %) from perennial ryegrass hay incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	1.3706	0.6853	5.71	0.010
Incubation time	3	15.3157	5.1052	42.51	< 0.001
Reading interval.Incubation time	6	0.9622	0.1604	1.34	0.284
Replicates	2	0.0238	0.0119	0.10	0.906
Residual	22	2.6420	0.1201		
Total	35	20.3144			

Appendix 4.2.1.21 The effect of reading bottles every 2, 4 or 6 h on the valerate production (molar %) from perennial ryegrass hay incubated with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	0.63412	0.31706	8.71	0.002
Incubation time	3	4.27083	1.42361	39.11	< 0.001
Reading interval.Incubation time	6	0.30353	0.05059	1.39	0.263
Replicates	2	0.12007	0.06003	1.65	0.215
Residual	22	0.80073	0.03640		
Total	35	6.12928			

Appendix 4.2.1.22 The effect of reading bottles every 2, 4 or 6 h on the resulting pH during incubation of 1.00 g naked oats with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	0.003150	0.001575	3.02	0.069
Incubation time	3	0.042189	0.014063	26.94	< 0.001
Reading interval.Incubation time	6	0.000828	0.000138	0.26	0.948
Replicates	2	0.000050	0.000025	0.05	0.953
Residual	22	0.011483	0.000522		
Total	35	0.057700			

Appendix 4.2.1.23 The effect of reading bottles every 2, 4 or 6 h on the resulting pH during incubation of 0.75 g hay with rumen micro-organisms at 12, 24, 36 and 40/42 h incubation.

Source of variation	d.f. (m.v)	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	0.001117	0.000558	0.82	0.455
Incubation time	3	0.041989	0.013996	20.46	< 0.001
Reading interval.Incubation time	6	0.023594	0.003932	5.75	0.001
Replicates	2	0.004550	0.002275	3.33	0.055
Residual	22	0.015050	0.000684		
Total	35	0.086300			

Appendix 4.2.2.1 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 1.00 g, 0.75 g, 0.50 g and 0.25 g naked oats incubated with a rumen microbial inoculum with gas production recorded, using the pressure transducer technique, every 2, 4 or 6 h; ignoring substrate weight.

Treatment	Source[1]	DF	SS	MS	F-ratio[2]
2 h data for all substrate weights	Between rates (b and c)	6	65.2939	10.8823	0.4559
	Between gas pool (A and B)	6	257065.7000	42844.2800	1794.92***
	within subset	63	1503.7940	23.8698	
4 h data for all substrate weights	Between rates (b and c)	6	78.4671	13.0779	2.2693
	Between gas pool (A and B)	6	115592.7000	19265.4500	3342.955***
	within subset	24	138.3121	5.7630	
6 h data for all substrate weights	Between rates (b and c)	6	72.1637	12.0273	6.5834**
	Between gas pool (A and B)	6	56434.6300	9405.7720	5148.4876***
	within subset	12	21.9233	1.8269	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. F values at the 5% levels were 2.254, 2.508 and 2.996 where v1=6 and v2=63, 24 or 12 respectively. For the 1 % level, values of F were 3.119, 3.667 and 4.821 whilst the 0.1 % values were 4.372, 5.550 and 8.379 for v1=6 and v2=63, 24 or 12 respectively. (Lindley & Scott, 1990).

Appendix 4.2.2.2 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 1.00 g, 0.75 g, 0.50 g and 0.25 g naked oats incubated with a rumen microbial inoculum with gas production recorded, using the pressure transducer technique, every 2, 4 or 6 h; the effect on individual substrate weights.

Treatment	Source[1]	DF	SS	MS	F-ratio[2]
0.25 g	Between rates (b and c)	4	17.5258	4.3814	3.0914*
	Between gas pool (A and B)	4	14.4588	3.6147	2.5504
	within subset	24	34.0158	1.4173	
0.50 g	Between rates (b and c)	4	87.1537	21.7884	2.8035*
	Between gas pool (A and B)	4	1056.8810	264.2202	33.9969***
	within subset	25	194.2966	7.7719	
0.75 g	Between rates (b and c)	4	149.8810	37.4702	1.8670
	Between gas pool (A and B)	4	3671.1240	917.7809	45.7297***
	within subset	25	501.7426	20.0697	
1.00 g	Between rates (b and c)	4	293.0919	73.2730	1.9613
	Between gas pool (A and B)	4	5353.7550	1338.4390	35.8260***
	within subset	25	933.9850	37.3594	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
*p < 0.05, ** p < 0.01, *** p < 0.001. The 5, 1 and 0.1 % values of F for v1 = 4 and v2 = 25 were 2.759, 4.177 and 6.493 respectively. Where v1 = 4 and v2 = 24, values for F at the 5, 1 and 0.1% levels were 2.776, 4.218 and 6.589 respectively (Lindley & Scott, 1990).

Appendix 4.2.2.3 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of 1.00 g, 0.75 g, 0.50 g and 0.25 g naked oats incubated with a rumen microbial inoculum with gas production recorded, using the pressure transducer technique, every 2, 4 or 6 h; the effect on individual substrate weights.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	0.147503	0.073751	549.77	< 0.001
Substrate weight	3	0.105230	0.035077	261.47	< 0.001
interval.weight	6	0.020685	0.003447	25.70	< 0.001
Replicate	2	0.000031	0.000015	0.12	0.891
Residual	22	0.002951	0.000134		
Total	35	0.276400			

Appendix 4.2.2.4 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of 1.00 g, 0.75 g, 0.50 g and 0.25 g naked oats incubated with a rumen microbial inoculum with gas production recorded, using the pressure transducer technique, every 2, 4 or 6 h; the effect on individual substrate weights.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	4.52091	2.26045	654.79	< 0.001
Substrate weight	3	2.78783	0.92928	269.19	< 0.001
interval.weight	6	0.74098	0.12350	35.77	< 0.001
Replicate	2	0.00094	0.00047	0.14	0.873
Residual	22	0.07595	0.00345		
Total	35	8.12661			

Appendix 4.2.2.5 The cumulative gas production, A (ml; France *et al.*, 1993) obtained during incubation of 1.00 g, 0.75 g, 0.50 g and 0.25 g naked oats incubated with a rumen microbial inoculum with gas production recorded, using the pressure transducer technique, every 2, 4 or 6 h; the effect on individual substrate weights.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	171.24	85.62	1.11	0.348
Substrate weight	3	141706.06	47235.35	610.51	< 0.001
interval.weight	6	4159.61	693.27	8.96	< 0.001
Replicate	2	524.87	262.43	3.39	0.052
Residual	22	1702.16	77.37		
Total	35	148263.94			

Appendix 4.2.2.6 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of 1.00 g, 0.75 g, 0.50 g and 0.25 g naked oats incubated with a rumen microbial inoculum with gas production recorded, using the pressure transducer technique, every 2, 4 or 6 h; the effect on individual substrate weights.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	144056.6	72028.3	169.16	< 0.001
Substrate weight	3	10055.6	3351.9	7.87	< 0.001
interval.weight	6	33067.2	5511.2	12.94	< 0.001
Replicate	2	981.4	490.7	1.15	0.334
Residual	22	9367.4	425.8		
Total	35	197528.3			

Appendix 4.2.2.7 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of 1.00 g, 0.75 g, 0.50 g and 0.25 g naked oats incubated with a rumen microbial inoculum with gas production recorded, using the pressure transducer technique, every 2, 4 or 6 h; the effect on individual substrate weights.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	1.89438	0.94719	11.73	< 0.001
Substrate weight	3	10.23589	3.41196	42.26	< 0.001
interval.weight	6	16.98411	2.83069	35.06	< 0.001
Replicate	2	0.14251	0.07125	0.88	0.428
Residual	22	1.77613	0.08073		
Total	35	31.03302			

Appendix 4.2.2.8 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of 1.00 g, 0.75 g, 0.50 g and 0.25 g naked oats incubated with a rumen microbial inoculum with gas production recorded, using the pressure transducer technique, every 2, 4 or 6 h; the effect on individual substrate weights.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	7.1495	3.5747	12.84	< 0.001
Substrate weight	3	6.1832	2.0611	7.41	0.001
interval.weight	6	8.4269	1.4045	5.05	0.002
Replicate	2	0.6951	0.3475	1.25	0.306
Residual	22	6.1229	0.2783		
Total	35	28.5775			

Appendix 4.2.2.9 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of 1.00 g, 0.75 g, 0.50 g and 0.25 g naked oats incubated with a rumen microbial inoculum with gas production recorded, using the pressure transducer technique, every 2, 4 or 6 h; the effect on individual substrate weights.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Reading interval	2	2883.88	1441.94	98.45	< 0.001
Substrate weight	3	2360.70	786.90	53.73	< 0.001
interval.weight	6	1715.84	285.97	19.52	< 0.001
Replicate	2	23.06	11.53	0.79	0.468
Residual	22	322.23	14.65		
Total	35	7305.71			

Appendix 4.2.4 The effect of reading bottles every 2, 4 or 6 h, using a dwell time of either 20, 60 or 120 s, on the resulting cumulative gas volume at 12 h incubation from 1.00 g naked oats incubated with rumen micro-organisms.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Reading interval (2,4, or 6 h)	2	16109.73	8054.87	255.18	< 0.001
Time (20, 60 or 120 s)	2	248.80	124.40	3.94	0.038
interval.time	4	128.61	32.15	1.02	0.424
Residual	18	568.18	31.57		
Total	26	17055.33			

Appendix 4.3.1 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 0.75 g perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum and either shaken continuously (series 1) after every gas reading (series 2) or not shaken (series 3) throughout the incubation.

Treatment	Source[1]	DF	SS	MS	F-ratio[2]
series 1 vs series 2	Between rates (b and c)	2	4.8902	2.4451	0.69
	Between gas pool (A and B)	2	299.1849	149.5924	42.22***
	within subset	26	92.1317	3.5435	
series 1 vs series 3	Between rates (b and c)	2	77.0826	38.5413	10.51***
	Between gas pool (A and B)	2	1321.1640	660.5820	180.09***
	within subset	26	95.3696	3.6681	
series 2 vs series 3	Between rates (b and c)	2	46.2705	23.1352	6.3304**
	Between gas pool (A and B)	2	363.4746	181.7373	49.7284***
	within subset	26	95.0207	3.6546	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
***$p < 0.001$, **$p < 0.01$. The F values were 5.526 and 9.116 at the 1 and 0.1 % levels respectively (Lindley & Scott, 1984).

Appendix 4.3.2 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained from 0.75 g perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum and either shaken continuously (series 1) after every gas reading (series 2) or not shaken (series 3) throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	0.00035	0.000175	41.18	0.002
Replicate	2	0.00002	0.000010	2.47	0.200
Residual	4	0.00002	0.000004		
Total	8	0.00039			

Appendix 4.3.3 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained from 0.75 g perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum and either shaken continuously (series 1) after every gas reading (series 2) or not shaken (series 3) throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	0.0162	0.00809	894.09	< 0.001
Replicate	2	0.000196	0.000098	10.84	0.024
Residual	4	0.000036	0.000009		
Total	8	0.0164			

Appendix 4.3.4 The cumulative gas production, A (ml; France *et al.*, 1993) obtained from 0.75 g perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum and either shaken continuously (series 1) after every gas reading (series 2) or not shaken (series 3) throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	335.85	167.93	12.92	0.018
Replicate	2	103.10	51.55	3.97	0.112
Residual	4	52.00	13.00		
Total	8	490.95			

Appendix 4.3.5 The gas production parameter, B, obtained from the France *et al.* (1993) model from 0.75 g perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum and either shaken continuously (series 1) after every gas reading (series 2) or not shaken (series 3) throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	1793.969	896.985	92.68	< 0.001
Replicate	2	110.886	55.443	5.73	0.067
Residual	4	38.715	9.679		
Total	8	1943.571			

Appendix 4.3.6 The lag time, L_T (h; France *et al.*, 1993) obtained from 0.75 g perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum and either shaken continuously (series 1) after every gas reading (series 2) or not shaken (series 3) throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	1.82290	0.91145	296.49	< 0.001
Replicate	2	0.01956	0.00978	3.18	0.149
Residual	4	0.01229	0.00307		
Total	8	1.85476			

Appendix 4.3.7 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained from 0.75 g perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum and either shaken continuously (series 1) after every gas reading (series 2) or not shaken (series 3) throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	3.4856	1.7428	4.34	0.099
Replicate	2	1.9277	0.9638	2.40	0.206
Residual	4	1.6052	0.4013		
Total	8	7.0184			

Appendix 4.3.8 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained from 0.75 g perennial ryegrass hay (*Lolium perenne*) incubated with a rumen microbial inoculum and either shaken continuously (series 1) after every gas reading (series 2) or not shaken (series 3) throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	14.052	7.026	1.33	0.362
Replicate	2	8.410	4.205	0.79	0.513
Residual	4	21.199	5.300		
Total	8	43.661			

Appendix 4.3.9 Dry matter loss from perennial ryegrass hay (*Lolium perenne*), incubated with a rumen microbial inoculum and subjected to continual shaking, shaking after every gas reading (normal) or not shaken throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	4556.5	2278.3	19.15	0.009
Replicate	2	116.0	058.0	0.49	0.646
Residual	4	475.9	119.0		
Total	8	5148.4			

Appendix 4.3.10 Acetate production (mmol l^{-1}) during 72 h incubation of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum, subjected to either continual shaking, shaking after every gas reading (normal) or not shaken throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	4.535	2.267	0.38	0.708
Replicate	2	65.343	32.672	5.43	0.072
Residual	4	24.049	6.012		
Total	8	93.927			

Appendix 4.3.11 Propionate production (mmol l^{-1}) during 72 h incubation of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum, subjected to either continual shaking, shaking after every gas reading (normal) or not shaken throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	0.665	0.333	0.29	0.760
Replicate	2	10.820	5.410	4.77	0.087
Residual	4	4.534	1.133		
Total	8	16.019			

Appendix 4.3.12 Butyrate production (mmol l^{-1}) during 72 h incubation of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum, subjected to either continual shaking, shaking after every gas reading (normal) or not shaken throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	1.3271	0.6635	4.09	0.108
Replicate	2	1.4390	0.7195	4.43	0.097
Residual	4	0.6490	0.1623		
Total	8	3.4151			

Appendix 4.3.13 Valerate production (mmol l^{-1}) during 72 h incubation of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum, subjected to either continual shaking, shaking after every gas reading (normal) or not shaken throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	0.15291	0.07646	2.19	0.228
Replicate	2	0.04921	0.02460	0.70	0.547
Residual	4	0.13965	0.03491		
Total	8	0.34176			

Appendix 4.3.14 Total volatile fatty acid (VFA) production (mmol l^{-1}) during 72 h incubation of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum, subjected to either continual shaking, shaking after every gas reading (normal) or not shaken throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	16.73	8.36	0.51	0.663
Replicate	2	162.60	81.30	5.00	0.082
Residual	4	65.05	16.26		
Total	8	244.38			

Appendix 4.3.15 pH after 72 h incubation of perennial ryegrass hay (*Lolium perenne*), with a rumen microbial inoculum and subjected to continual shaking, shaking after every gas reading (normal) or not shaken throughout the incubation.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Shaking treatment	2	0.0092	0.0046	8.16	0.039
Replicate	2	0.0004	0.0002	0.38	0.708
Residual	4	0.0022	0.0006		
Total	8	0.0118			

APPENDIX 5

Appendix 5.1.1 Parallel curve analysis of the gas production profiles obtained during incubation of 1.0 g oatfeed, naked oats or soya hulls with a rumen microbial inoculum.

Substrate	Source[1]	DF	SS	MS	F-ratio[2]
Oatfeed vs soya hulls	Between rates (b and c)	2	701.9256	350.9628	28.29***
	Between gas pool (A and B)	2	118209.3000	59104.6600	4764.43***
	within subset	20	248.1084	12.4054	
Oatfeed vs naked oats	Between rates (b and c)	2	5598.8050	2799.4030	110.34***
	Between gas pool (A and B)	2	111099.1000	55549.5300	2189.57***
	within subset	20	507.3990	25.3700	
Naked oats vs soya hulls	Between rates (b and c)	2	6399.9570	3199.9790	145.17***
	Between gas pool (A and B)	2	3317.5960	1658.7980	75.25***
	within subset	20	440.8651	22.0433	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

*p < 0.05, **p < 0.01, ***p < 0.001. The F values at the 5, 1 and 0.1 % levels were 3.493, 5.849 and 9.953, respectively at v1 = 2 and v2 = 20 degrees of freedom (df). (Lindley & Scott, 1990).

Appendix 5.1.2.1 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of 0.2, 0.4, 0.6, 0.8 and 1.0 g of naked oats, oatfeed and soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	0.3054374	0.1527187	2739.20	< 0.001
weight	4	0.0042655	0.0010664	19.13	< 0.001
substrate.weight	8	0.0078516	0.0009814	17.60	< 0.001
Replicate	2	0.0000676	0.0000338	0.61	0.552
Residual	28	0.0015611	0.0000558		
Total	44	0.3191832			

Appendix 5.1.2.2 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of 0.2, 0.4, 0.6, 0.8 and 1.0 g of naked oats, oatfeed and soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	4.855046	2.4275.23	2293.79	< 0.001
weight	4	0.132844	0.033211	31.38	< 0.001
substrate.weight	8	0.178177	0.022272	21.05	< 0.001
Replicate	2	0.001142	0.000571	0.54	0.589
Residual	28	0.029632	0.001058		
Total	44	5.196842			

Appendix 5.1.2.3 The cumulative gas production, A (ml; France *et al.*, 1993) obtained during incubation of 0.2, 0.4, 0.6, 0.8 and 1.0 g of naked oats, oatfeed and soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	83929.30	41964.65	4774.58	< 0.001
weight	4	211520.43	52880.11	6016.49	< 0.001
substrate.weight	8	17848.64	2231.08	253.84	< 0.001
Replicate	2	8.03	4.01	0.46	0.638
Residual	28	246.10	8.79		
Total	44	313552.50			

Appendix 5.1.2.4 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of 0.2, 0.4, 0.6, 0.8 and 1.0 g of naked oats, oatfeed and soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	51833.50	25916.75	1290.68	< 0.001
weight	4	90017.29	22504.32	1120.74	< 0.001
substrate.weight	8	12477.24	1559.65	77.67	< 0.001
Replicate	2	38.61	19.31	0.96	0.395
Residual	28	562.24	20.08		
Total	44	154928.87			

Appendix 5.1.2.5 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of 0.2, 0.4, 0.6, 0.8 and 1.0 g of naked oats, oatfeed and soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	11.09335	5.54668	251.39	< 0.001
weight	4	3.40176	0.85044	38.54	< 0.001
substrate.weight	8	6.54875	0.81859	37.10	< 0.001
Replicate	2	0.03948	0.01974	0.89	0.420
Residual	28	0.61780	0.02206		
Total	44	21.70114			

Appendix 5.1.2.6 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of 0.2, 0.4, 0.6, 0.8 and 1.0 g of naked oats, oatfeed and soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	838.1555	419.0777	3404.93	< 0.001
weight	4	35.4408	8.8602	71.99	< 0.001
substrate.weight	8	36.0999	4.5125	36.66	< 0.001
Replicate	2	0.1726	0.0863	0.70	0.505
Residual	28	3.4462	0.1231		
Total	44	913.3149			

Appendix 5.1.2.7 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of 0.2, 0.4, 0.6, 0.8 and 1.0 g of naked oats, oatfeed and soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	14840.070	7420.035	0.0001	< 0.001
weight	4	58.806	14.701	23.23	< 0.001
substrate.weight	8	136.995	17.124	27.06	< 0.001
Replicate	2	0.180	0.090	0.14	0.868
Residual	28	17.722	0.633		
Total	44	15053.773			

Appendix 5.1.3.1 Parallel curve analysis of the DM loss from 1.0 g oatfeed or naked oats during incubation with a rumen microbial inoculum.

Substrate	Source[1]	DF	SS	MS	F-ratio[2]
Oatfeed vs naked oats	Between rates (b and c)	2	15326.0100	7663.0070	4.3087*
	Between gas pool (A and B)	2	1842359.0000	921179.3000	517.9580***
	within subset	14	24898.7500	1778.4820	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

*p < 0.05, **p < 0.01, ***p < 0.001. The F values at the 5, 1 and 0.1 % levels were 3.739, 6.515 and 11.78, respectively at v1 = 2 and v2 = 14 degrees of freedom (df). (Lindley & Scott, 1990).

Appendix 5.1.3.2 Dry matter loss from naked oats, oatfeed and soya hulls after 60 h incubation with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	3033200.9	1516600.5	2969.23	< 0.001
Weight	4	2599.7	649.9	1.27	0.304
Substrate.weight	8	4356.4	544.6	1.07	0.414
Replicate	2	904.9	452.4	0.89	0.424
Residual	28	14301.6	510.8		
Total	44	3055363.5			

Appendix 5.1.4 Parallel curve analysis of the volatile fatty acid (VFA) production profiles; acetate, propionate and butyrate, obtained during incubation of 1.0 g oatfeed or naked oats with a rumen microbial inoculum.

VFA	Source[1]	DF	SS	MS	F-ratio[2]
Acetate production	Between rates (b and c)	2	56.8607	28.4303	18.9801***
	Between gas pool (A and B)	2	680.6125	340.3063	227.1889***
	within subset	12	17.9743	1.4979	
Propionate production	Between rates (b and c)	2	2.4397	1.2199	1.6645
	Between gas pool (A and B)	2	1746.4380	873.2190	1191.4572***
	within subset	12	8.7942	0.7329	
Butyrate production	Between rates (b and c)	2	2323.7990	1161.9000	2.5846
	Between gas pool (A and B)	2	335035.3000	167517.6000	372.6345***
	within subset	12	5394.5730	449.5477	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

*p < 0.05, **p < 0.01, ***p < 0.001. The F values at the 5, 1 and 0.1 % levels were 3.885, 6.927 and 12.970, respectively at v1 = 2 and v2 = 12 degrees of freedom (df). (Lindley & Scott, 1990).

Appendix 5.1.5.1 Total volatile fatty acid (VFA) production (mmol l^{-1}) following 60 h incubation of naked oats, oatfeed or soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	2870.045	1435.023	161.08	< 0.001
Replicate	2	10.836	5.418	0.61	0.588
Residual	4	35.636	8.909		
Total	8	2916.518			

Appendix 5.1.5.2 Acetate production (molar %) following 60 h incubation of naked oats, oatfeed or soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	1443.1437	721.5718	2789.14	< 0.001
Replicate	2	0.0533	0.0266	0.10	0.904
Residual	4	1.0348	0.2587		
Total	8	1444.2318			

Appendix 5.1.5.3 Propionate production (molar %) following 60 h incubation of naked oats, oatfeed or soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	1173.8564	586.9282	2910.58	< 0.001
Replicate	2	0.0299	0.0150	0.07	0.930
Residual	4	0.8066	0.2017		
Total	8	1174.6929			

Appendix 5.1.5.4 Butyrate production (molar %) following 60 h incubation of naked oats, oatfeed or soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	9.73254	4.86627	146.43	< 0.001
Replicate	2	0.12321	0.06160	1.85	0.269
Residual	4	0.13293	0.03323		
Total	8	9.98868			

Appendix 5.1.5.5 Valerate production (molar %) following 60 h incubation of naked oats, oatfeed or soya hulls with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	1.045870	0.522935	159.37	< 0.001
Replicate	2	0.007172	0.003586	1.09	0.418
Residual	4	0.013125	0.003281		
Total	8	1.066167			

Appendix 5.1.6 pH following 60 h incubation of naked oats, oatfeed and soya hulls with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Substrate	2	0.018422	0.009211	4.70	0.089
Replicate	2	0.001622	0.000811	0.41	0.687
Residual	4	0.007844	0.001961		
Total	8	0.027889			

Appendix 5.1.7 Parallel curve analysis of the gas production profiles obtained during stepwise addition of 2 ml of 1 mol l^{-1} acetic, propionic or butyric acid to the culture medium used in gas production studies.

	Source[1]	DF	SS	MS	F-ratio[2]
Ac vs Pr vs Bu	Between rates (b and c)	4	50.5228	12.6307	1.1711
	Between gas pool (A and B)	4	91.8680	22.9670	2.1294
	within subset	24	42.8535	10.7856	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
*p < 0.05, **p < 0.01, ***p < 0.001. The F values at the 5, 1 and 0.1 % levels were 2.776, 4.218 and 6.589, respectively at v1 = 4 and v2 = 24 degrees of freedom (df). (Lindley & Scott, 1990).

Appendix 5.1.8 Parallel curve analysis of the pH profiles obtained during stepwise addition of 2 ml of 1 mol l^{-1} acetic, propionic or butyric acid to the culture medium used in gas production studies.

	Source[1]	DF	SS	MS	F-ratio[2]
Ac vs Pr vs Bu	Between rates (b and c)	4	0.0627	0.0130	2.708
	Between gas pool (A and B)	4	0.0310	0.0078	1.625
	within subset	24	0.1154	0.0048	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
*p < 0.05, **p < 0.01, ***p < 0.001. The F values at the 5, 1 and 0.1 % levels were 2.776, 4.218 and 6.589, respectively at v1 = 4 and v2 = 24 degrees of freedom (df). (Lindley & Scott, 1990).

Appendix 5.1.9 Parallel curve analysis of the gas production profiles obtained during incubation of grass, clover and various grass / clover mixtures with a rumen microbial inoculum.

Substrate	Source[1]	DF	SS	MS	F-ratio[2]
grass versus clover	Between rates (b and c)	2	21.2538	10.6269	0.9073
	Between gas pool (A and B)	2	20015.9200	10007.9600	854.4829***
	within subset	24	281.0958	11.7123	
grass versus 80:20 G:C	Between rates (b and c)	2	34.0605	17.0303	1.1504
	Between gas pool (A and B)	2	765.0440	382.5220	25.8398***
	within subset	24	355.2869	14.8036	
grass versus 60:40 G:C	Between rates (b and c)	2	78.2160	39.1080	2.8205
	Between gas pool (A and B)	2	2127.8340	1063.9170	76.7301***
	within subset	24	332.7773	13.8657	
grass versus 40:60 G:C	Between rates (b and c)	2	92.0417	46.0209	3.5734*
	Between gas pool (A and B)	2	4361.9030	2180.9520	169.3457***
	within subset	24	309.0880	12.8787	
grass versus 20:80 G:C	Between rates (b and c)	2	118.0812	59.0406	5.3091*
	Between gas pool (A and B)	2	8466.0530	4233.0260	380.6473***
	within subset	24	266.8953	11.1206	
clover versus 80:20 G:C	Between rates (b and c)	2	1.2931	0.6465	0.0581
	Between gas pool (A and B)	2	13060.3600	6530.1800	586.5027***
	within subset	24	267.2191	11.1341	
clover versus 60:40 G:C	Between rates (b and c)	2	12.8437	6.4219	0.6298
	Between gas pool (A and B)	2	9245.2020	4622.6010	453.3517***
	within subset	24	244.7157	10.1965	
clover versus 40:60 G:C	Between rates (b and c)	2	18.7452	9.3726	1.0177
	Between gas pool (A and B)	2	5830.0880	2915.0440	316.5223***
	within subset	24	221.0300	9.2096	
	Between rates (b and c)	2	31.2012	15.6006	2.0936

clover versus 20:80 G:C	Between gas pool (A and B)	2	2508.1030	1254.0520	168.2929***
	within subset	24	178.8376	7.4516	
80:20 versus 20:80 G:C	Between rates (b and c)	2	30.5613	15.2807	1.4494
	Between gas pool (A and B)	2	4164.0020	2082.0010	197.4827***
	within subset	24	253.0253	10.5427	
80:20 versus 60:40 G:C	Between rates (b and c)	2	9.5805	4.7903	0.3605
	Between gas pool (A and B)	2	341.1572	170.5786	12.8372***
	within subset	24	318.9062	13.2878	
80:20 versus 40:60 G:C	Between rates (b and c)	2	15.8568	7.9284	0.6445
	Between gas pool (A and B)	2	1476.3690	738.1844	60.0106***
	within subset	24	295.2209	12.3009	
60:40 versus 40:60 G:C	Between rates (b and c)	2	0.8928	0.4464	0.0393
	Between gas pool (A and B)	2	399.5385	199.7692	17.5808***
	within subset	24	272.7090	11.3629	
60:40 versus 20:80 G:C	Between rates (b and c)	2	7.1213	3.5607	0.3707
	Between gas pool (A and B)	2	2135.6030	1067.8010	111.1737***
	within subset	24	230.5152	9.6048	
40:60 versus 20:80 G:C	Between rates (b and c)	2	3.0501	1.5251	0.1770
	Between gas pool (A and B)	2	691.8331	345.9166	40.1402***
	within subset	24	206.8257	8.6177	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. The F values at the 5, 1 and 0.1 % levels were 3.403, 5.614 and 9.339, respectively at 24 degrees of freedom (df). (Lindley & Scott, 1990).

Appendix 5.1.10.1 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of 100 % grass, 100 % clover and various grass:clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Mixture	5	0.0000927	0.0000185	1.31	0.335
Replicate	2	0.0000196	0.0000098	0.69	0.524
Residual	10	0.0001419	0.0000142		
Total	17	0.0002542			

Appendix 5.1.10.2 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of 100 % grass, 100 % clover and various grass:clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Mixture	5	0.009797	0.001959	3.12	0.059
Replicate	2	0.000753	0.000377	0.60	0.567
Residual	10	0.006273	0.000627		
Total	17	0.016823			

Appendix 5.1.10.3 The cumulative gas production, A (ml; France *et al.*, 1993) obtained during incubation of 100 % grass, 100 % clover and various grass:clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Mixture	5	8044.34	1608.87	21.59	< 0.001
Replicate	2	120.32	60.16	0.81	0.473
Residual	10	745.07	74.51		
Total	17	8909.72			

Appendix 5.1.10.4 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of 100 % grass, 100 % clover and various grass:clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Mixture	5	9568.7	1913.7	10.20	0.001
Replicate	2	293.8	146.9	0.78	0.483
Residual	10	1875.6	187.6		
Total	17	11738.0			

Appendix 5.1.10.5 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of 100 % grass, 100 % clover and various grass:clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Mixture	5	0.465393	0.093079	39.00	< 0.001
Replicate	2	0.010067	0.005034	2.11	0.172
Residual	10	0.023868	0.002387		
Total	17	0.499328			

Appendix 5.1.10.6 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of 100 % grass, 100 % clover and various grass:clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Mixture	5	2.77969	0.55594	12.17	< 0.001
Replicate	2	0.00404	0.00202	0.04	0.957
Residual	10	0.45676	0.04568		
Total	17	3.24049			

Appendix 5.1.10.7 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of 100 % grass, 100 % clover and various grass:clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Mixture	5	54.919	10.984	6.60	0.006
Replicate	2	1.975	0.988	0.59	0.571
Residual	10	16.637	1.664		
Total	17	73.531			

Appendix 5.1.11 Parallel curve analysis of the additive gas production profiles obtained during incubation of grass plus clover, and various grass / clover mixtures with a rumen microbial inoculum.

Substrate	Source[1]	DF	SS	MS	F-ratio[2]
grass+clover vs 80:20 + 20:80 G:C	Between rates (b and c)	2	133.8733	66.9366	1.6888
	Between gas pool (A and B)	2	618.6577	309.3289	7.8045**
	within subset	24	951.2333	39.6347	
grass+clover vs 60:40 + 40:60 G:C	Between rates (b and c)	2	181.0344	90.5172	2.1139
	Between gas pool (A and B)	2	1148.3880	574.1940	13.4099***
	within subset	24	1027.6470	42.8186	
80:20+20:80 G:Cvs 60:40 +40:60 G:C	Between rates (b and c)	2	4.4048	2.2024	0.0523
	Between gas pool (A and B)	2	81.3601	40.6801	0.9657
	within subset	24	1010.9860	42.1244	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

**p < 0.01, ***p < 0.001. The F values at the 5, 1 and 0.1 % levels were 3.403, 5.614 and 9.339, respectively at 24 degrees of freedom (df). (Lindley & Scott, 1990).

Appendix 5.1.12 Dry matter loss from perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*) and various ryegrass clover mixtures after incubation with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	5	11430.3	2286.1	8.97	0.002
Replicate	2	1611.3	805.7	3.16	0.086
Residual	10	2549.3	254.9		
Total	17	15591.0			

Appendix 5.1.13.1 Total volatile fatty acid (VFA) production (mmol l^{-1}) following incubation of perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*) and various ryegrass clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	5	149.86	29.97	1.95	0.172
Replicate	2	7.97	3.99	0.26	0.776
Residual	10	153.42	15.34		
Total	17	311.25			

Appendix 5.1.13.2 Acetate production (molar %) following incubation of perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*) and various ryegrass clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	5	29.5375	5.9075	8.59	0.002
Replicate	2	0.3477	0.1738	0.25	0.781
Residual	10	6.8788	0.6879		
Total	17	36.7640			

Appendix 5.1.13.3 Propionate production (molar %) following incubation of perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*) and various ryegrass clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	5	109.7074	21.9415	40.20	< 0.001
Replicate	2	0.3685	0.1842	0.34	0.721
Residual	10	5.4579	0.5458		
Total	17	115.5338			

Appendix 5.1.13.4 Butyrate production (molar %) following incubation of perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*) and various ryegrass clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	5	0.49514	0.09903	1.24	0.361
Replicate	2	0.09692	0.04846	0.61	0.565
Residual	10	0.799999	0.08000		
Total	17	1.39205			

Appendix 5.1.13.5 Valerate production (molar %) following incubation of perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*) and various ryegrass clover mixtures with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	5	23.0342	4.6068	37.44	< 0.001
Replicate	2	0.0040	0.0020	0.02	0.984
Residual	10	1.2304	0.1230		
Total	17	24.2686			

Appendix 5.1.14 pH following incubation of perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*) and various ryegrass clover mixtures with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Substrate	5	0.607983	0.121597	21.90	< 0.001
Replicate	2	0.065724	0.032862	5.92	0.023
Residual	9 (1)	0.049962	0.005551		
Total	16 (1)	0.602824			

Appendix 5.2.1.1 Parallel curve analysis of the gas production profiles obtained during incubation of the soluble fraction of hay or the whole hay sample with a rumen microbial inoculum.

Substrate	Source[1]	DF	SS	MS	F-ratio[2]
hay vs soluble hay	Between rates (b and c)	2	847.8911	423.9456	93.18***
	Between gas pool (A and B)	2	118243.4000	59121.6900	12994.06***
	within subset	34	154.6967	4.5499	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
***$p < 0.001$. The F value at the 0.1 % level was 8.522 for 34 degrees of freedom (df). (Lindley & Scott, 1990).

Appendix 5.2.1.2 Parallel curve analysis of the gas production profiles obtained during incubation of the soluble fraction of naked oats or the whole naked oat sample with a rumen microbial inoculum.

Substrate	Source[1]	DF	SS	MS	F-ratio[2]
NO vs soluble NO	Between rates (b and c)	2	223.0496	111.5248	2.29
	Between gas pool (A and B)	2	51419.1600	25709.5800	527.63***
	within subset	33	1607.9850	48.7268	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
***p < 0.001. The F value was 8.639, 5.336 and 3.295 at the 0.1, 1 and 5 % levels, respectively, for 33 degrees of freedom (df). (Lindley & Scott, 1990).

Appendix 5.2.1.3 Parallel curve analysis of the gas production profiles obtained during incubation of the soluble fraction of naked oats or the soluble fraction of hay and the whole naked oat sample or the whole hay sample with a rumen microbial inoculum.

Substrate	Source[1]	DF	SS	MS	F-ratio[2]
NO versus hay	Between rates (b and c)	2	10645.6500	5322.8250	136.08***
	Between gas pool (A and B)	2	20784.1300	10392.0600	265.69***
	within subset	34	1329.8710	39.1139	
soluble NO vs soluble hay	Between rates (b and c)	2	912.0641	456.0321	34.77***
	Between gas pool (A and B)	2	67723.8200	33861.9100	2581.84***
	within subset	33	432.8089	13.1154	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
***p < 0.001. The F values were 8.639, 5.336 and 3.295 at the 0.1, 1 and 5 % levels, respectively, for 33 degrees of freedom (df) whilst for 34 df the F values were 8.522, 5.289 and 3.276 at the 0.1, 1 and 5 % levels, respectively (Lindley & Scott, 1990).

Appendix 5.2.2.1 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of hay, naked oats and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	0.3103098	0.1034366	235.27	<0.001
Replicate	2	0.0007943	0.0003972	0.90	0.454
Residual	6	0.0026379	0.0004396		
Total	11	0.3137420			

Appendix 5.2.2.2 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of hay, naked oats and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	5.48852	1.82951	148.20	<0.001
Replicate	2	0.01965	0.00983	0.80	0.494
Residual	6	0.07407	0.01235		
Total	11	5.58224			

Appendix 5.2.2.3 The cumulative gas production, A (ml; France *et al.*, 1993) obtained during incubation of hay, naked oats and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	75376.68	25125.56	1046.29	<0.001
Replicate	2	46.20	23.10	0.96	0.434
Residual	6	144.08	24.01		
Total	11	75566.97			

Appendix 5.2.2.4 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of hay, naked oats and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	65381.90	21793.97	508.87	<0.001
Replicate	2	265.38	132.69	3.10	0.119
Residual	6	256.97	42.83		
Total	11	65904.24			

Appendix 5.2.2.5 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of hay, naked oats and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	13.17468	4.39156	125.22	<0.001
Replicate	2	0.02253	0.01126	0.32	0.737
Residual	6	0.21043	0.03507		
Total	11	13.40763			

Appendix 5.2.2.6 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of hay, naked oats and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	147.3826	49.1275	197.61	<0.001
Replicate	2	0.0954	0.0477	0.19	0.830
Residual	6	1.4916	0.2486		
Total	11	148.9697			

Appendix 5.2.2.7 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of hay, naked oats and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	3681.5513	1227.1838	2410.83	<0.001
Replicate	2	0.2332	0.1166	0.23	0.802
Residual	6	3.0542	0.5090		
Total	11	3684.8387			

Appendix 5.2.3 Dry matter loss from perennial ryegrass hay (*Lolium perenne*) and naked oats (*Avena nuda*) after 15 h in Van Soest medium (to obtain the soluble fraction) or 72 h incubation with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	690617.7	230205.9	719.31	< 0.001
Replicate	2	532.1	266.1	0.83	0.480
Residual	6	1920.2	320.0		
Total	11	693070.1			

Appendix 5.2.4.1 Total volatile fatty acid (VFA) production (mmol l^{-1}) following incubation of perennial ryegrass hay (*Lolium perenne*), naked oats (*Avena nuda*) and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	4803.80	1601.27	77.14	< 0.001
Replicate	2	45.46	22.73	1.10	0.393
Residual	6	124.55	20.76		
Total	11	4973.81			

Appendix 5.2.4.2 Acetate production (molar %) following incubation of perennial ryegrass hay (*Lolium perenne*), naked oats (*Avena nuda*) and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	466.989	155.663	83.85	< 0.001
Replicate	2	1.504	0.752	0.41	0.684
Residual	6	11.139	1.857		
Total	11	479.632			

Appendix 5.2.4.3 Propionate production (molar %) following incubation of perennial ryegrass hay (*Lolium perenne*), naked oats (*Avena nuda*) and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	370.8506	123.6169	243.57	< 0.001
Replicate	2	1.7439	0.8720	1.72	0.257
Residual	6	3.0451	0.5075		
Total	11	375.6396			

Appendix 5.2.4.4 Butyrate production (molar %) following incubation of perennial ryegrass hay (*Lolium perenne*), naked oats (*Avena nuda*) and the soluble fractions of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	16.0949	5.3650	28.37	< 0.001
Replicate	2	1.3674	0.6837	3.62	0.093
Residual	6	1.1346	0.1891		
Total	11	18.5969			

Appendix 5.2.4.5 Valerate production (molar %) following incubation of perennial ryegrass hay (*Lolium perenne*), naked oats (*Avena nuda*) and the soluble fraction of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	2.8555	0.9518	6.44	0.026
Replicate	2	0.0852	0.0426	0.29	0.760
Residual	6	0.8874	0.1479		
Total	11	3.8281			

Appendix 5.2.5 pH following incubation of perennial ryegrass hay (*Lolium perenne*), naked oats (*Avena nuda*) and the soluble fraction of both hay and naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	3	0.2884250	0.0961417	887.46	< 0.001
Replicate	2	0.0022167	0.0011083	10.23	0.012
Residual	6	0.0006500	0.0001083		
Total	11	0.2912917			

Appendix 5.2.6.1 Volatile fatty acid production (mmol l^{-1}) from the fermentation of unfractionated alfalfa and brome and their respective soluble and insoluble fractions, as reported by Stefanon *et al.* (1996).

Forgae	Fraction and growth stage		Acetate	Propionate	Butyrate	Total VFA
Alfalfa	Unfractionated	1	37.0	18.3	2.9	58.2
		2	39.3	17.9	2.9	60.1
		3	37.0	16.5	2.7	56.2
		4	37.7	16.8	2.6	57.2
		5	33.2	14.4	2.5	50.0
	Insoluble fraction	1	33.6	16.7	1.9	52.3
		2	35.7	15.0	2.3	52.9
		3	36.4	14.6	2.1	53.1
		4	33.0	15.3	1.8	50.1
		5	31.3	12.7	1.8	45.8
	Soluble fraction	1	11.8	7.1	0.5	19.3
		2	11.7	6.5	0.4	18.5
		3	13.1	6.8	0.5	20.4
		4	13.0	7.2	0.6	20.7
		5	10.5	5.7	0.4	16.6
Brome	Unfractionated	1	38.8	15.1	3.4	57.2
		2	45.4	18.9	4.4	68.8
		3	44.4	16.4	3.6	64.4
		4	46.1	16.6	3.4	66.1
		5	44.7	16.7	3.4	64.9
	Insoluble fraction	1	42.1	16.3	3.4	61.7
		2	48.6	18.5	4.3	71.4
		3	44.7	17.6	3.6	65.9
		4	35.9	14.8	2.9	53.6
		5	37.2	15.7	3.0	55.8
	Soluble fraction	1	11.2	4.6	0.7	16.5
		2	11.6	6.4	1.0	19.0
		3	10.2	4.7	0.6	15.6
		4	10.6	4.6	0.5	15.8
		5	9.2	4.1	0.6	14.5

Appendix 5.2.6.2 Acetate production (molar %) from unfractionated samples of alfalfa and brome grass and their respective soluble and insoluble fractions (data from Stefanon *et al.*, 1996).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	1	25.682	25.682	9.53	0.006
Fraction	2	64.250	32.125	11.91	<0.001
Substrate.Fraction	2	6.170	3.085	1.14	0.338
Growth stage	4	10.280	2.570	0.95	0.454
Residual	20	53.924	2.696		
Total	29	160.306			

Appendix 5.2.6.3 Propionate production (molar %) from unfractionated samples of alfalfa and brome grass and their respective soluble and insoluble fractions (data from Stefanon *et al.*, 1996).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	1	101.338	101.338	46.49	<0.001
Fraction	2	126.743	63.371	29.08	<0.001
Substrate.Fraction	2	9.846	4.923	2.26	0.130
Growth stage	4	10.315	2.579	1.18	0.348
Residual	20	43.591	2.180		
Total	29	291.834			

Appendix 5.2.6.4 Butyrate production (molar %) from unfractionated samples of alfalfa and brome grass and their respective soluble and insoluble fractions (data from Stefanon *et al.*, 1996).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	1	14.1753	14.1753	100.56	<0.001
Fraction	2	19.9357	9.9678	70.72	<0.001
Substrate.Fraction	2	1.1109	0.5554	3.94	0.036
Growth stage	4	1.6379	0.4095	2.91	0.048
Residual	20	2.8189	0.1409		
Total	29	39.6768			

Appendix 5.3.1 Parallel curve analysis of the gas production profiles obtained during incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal (EC), equine faecal (EF) or a bovine rumen (BR) microbial inoculum.

Substrate	Source[1]	DF	SS	MS	F-ratio[2]
Oatfeed EC vs EF	Between rates (b and c)	2	43.0193	21.5096	41.3249***
	Between gas pool (A and B)	2	113.1342	56.5671	108.6784***
	within subset	19	9.8893	0.5205	
Oatfeed EC vs BR	Between rates (b and c)	2	0.9875	0.4938	0.6707
	Between gas pool (A and B)	2	4837.9980	2418.9990	3285.7905***
	within subset	20	14.7235	0.7362	
Oatfeed BR vs EF	Between rates (b and c)	2	51.6015	25.8007	25.3271***
	Between gas pool (A and B)	2	6128.1320	3064.0660	3007.8198***
	within subset	19	19.3559	1.0187	
Naked oats EC vs EF	Between rates (b and c)	2	1201.8500	600.9250	3.7993*
	Between gas pool (A and B)	2	347.9448	173.9724	1.0999
	within subset	20	3163.3180	158.1659	
	Between rates	2	7572.6650	3786.3330	29.3871***

Naked oats EC vs BR	(b and c)				
	Between gas pool (A and B)	2	5119.6520	2559.8260	19.8677***
	within subset	20	2576.8660	128.8433	
Naked oats EF vs BR	Between rates (b and c)	2	3629.8210	1814.9100	49.2029***
	Between gas pool (A and B)	2	2874.9390	1437.4690	38.9704***
	within subset	20	737.7247	36.8862	
Soya hulls EC vs EF	Between rates (b and c)	2	85.9854	42.9927	3.9921*
	Between gas pool (A and B)	2	181.6833	90.8417	8.4351**
	within subset	20	215.3910	10.7695	
Soya hulls EC vs BR	Between rates (b and c)	2	45.2713	22.6356	2.0629
	Between gas pool (A and B)	2	3243.4260	1621.7130	147.7966***
	within subset	20	219.4518	10.9726	
Soya hulls EF vs BR	Between rates (b and c)	2	233.8263	116.9131	7.3562**
	Between gas pool (A and B)	2	2864.6130	1432.3060	90.1212***
	within subset	20	317.8615	15.8931	
Sugar beet EC vs EF	Between rates (b and c)	2	106.1648	53.0824	3.3122
	Between gas pool (A and B)	2	20.5118	10.2559	0.6399
	within subset	19	304.5028	16.0265	
Sugar beet EC vs BR	Between rates (b and c)	2	157.3062	78.6531	4.6153*
	Between gas pool (A and B)	2	4521.4440	2260.7220	132.6590***
	within subset	20	340.8328	17.0416	
Sugar beet EF vs BR	Between rates (b and c)	2	62.9902	31.4951	7.4867**
	Between gas pool (A and B)	2	5053.4740	2526.7370	600.6316***
	within subset	19	79.9289	4.2068	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. The F values were 10.160, 5.926 and 3.522 at the 0.1, 1 and 5 % levels, respectively, for v1 = 2 and v2 = 19 degrees of freedom (df) whilst for v1 = 2 and v2 = 20 df the F values were 9.953, 5.849 and 3.493 at the 0.1, 1 and 5 % levels, respectively (Lindley & Scott, 1990).

Appendix 5.3.2.1 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen fluid inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	0.083956	0.041978	269.37	<0.001
Inocula	2	0.008588	0.004294	27.55	<0.001
substrate.inocula	4	0.019754	0.004938	31.69	<0.001
Replicate	3	0.000196	0.000065	0.42	0.740
Residual	24	0.003740	0.000156		
Total	35	0.116234			

Appendix 5.3.2.2 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen fluid inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	2.36918	1.18459	232.81	<0.001
Inocula	2	0.11438	0.05719	11.24	<0.001
substrate.inocula	4	0.27857	0.06964	13.69	<0.001
Replicate	3	0.01033	0.00344	0.68	0.575
Residual	24	0.12212	0.00509		
Total	35	2.89458			

Appendix 5.3.2.3 The cumulative gas production, A (ml; France *et al.*, 1993) obtained during incubation of naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen fluid inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	4045.96	2022.98	64.25	<0.001
Inocula	2	2618.99	1309.50	41.59	<0.001
substrate.inocula	4	10072.80	2518.20	79.98	<0.001
Replicate	3	152.23	50.74	1.61	0.213
Residual	24	755.62	31.48		
Total	35	17645.61			

Appendix 5.3.2.4 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen fluid inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	61364.36	30682.18	1069.71	<0.001
Inocula	2	7641.98	3820.99	133.22	<0.001
substrate.inocula	4	9890.36	2472.59	86.20	<0.001
Replicate	3	78.44	26.15	0.91	0.450
Residual	24	688.39	28.68		
Total	35	79663.52			

Appendix 5.3.2.5 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen fluid inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	65.28417	32.64209	501.63	<0.001
Inocula	2	9.05830	4.52915	69.60	<0.001
substrate.inocula	4	44.77573	11.19393	172.02	<0.001
Replicate	3	0.08152	0.02717	0.42	0.742
Residual	24	1.56174	0.06507		
Total	35	120.76146			

Appendix 5.3.2.6 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen fluid inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	215.986	107.993	94.52	<0.001
Inocula	2	144.898	72.449	63.41	<0.001
substrate.inocula	4	297.648	74.412	65.13	<0.001
Replicate	3	3.249	1.083	0.95	0.433
Residual	24	27.422	1.143		
Total	35	689.203			

Appendix 5.3.2.7 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen fluid inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Substrate	2	8216.845	4108.422	493.31	<0.001
Inocula	2	1074.422	537.211	64.50	<0.001
substrate.inocula	4	1187.474	296.869	35.65	<0.001
Replicate	3	18.101	6.034	0.72	0.547
Residual	24	199.877	8.328		
Total	35	10696.719			

Appendix 5.3.3 Dry matter loss from oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp after incubation with either an equine caecal, equine faecal or bovine rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Inocula	2	90594.41	45297.20	807.78	< 0.001
Feed	3	2809996.19	936665.40	0.0000167	< 0.001
Inocula.Feed	6	37813.55	6302.26	112.39	< 0.001
Replicate	3	486.56	162.19	2.89	0.050
Residual	33	1850.51	056.08		
Total	47	2940741.22			

Appendix 5.3.4.1 Total volatile fatty acid (VFA) production (mmol l^{-1}) following incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Inocula	2	315.258	157.629	36.56	< 0.001
Feed	3	7698.780	2566.260	595.27	< 0.001
Inocula.Feed	6	178.944	29.824	6.92	< 0.001
Replicate	3	14.945	4.982	1.16	0.342
Residual	32 (1)	137.955	4.311		
Total	46 (1)	8312.762			

Appendix 5.3.4.2 Acetate production (molar %) following incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Inocula	2	517.630	258.815	144.31	< 0.001
Feed	3	5163.456	1721.152	959.71	< 0.001
Inocula.Feed	6	635.511	105.918	59.06	< 0.001
Replicate	3	9.287	3.096	1.73	0.181
Residual	32 (1)	57.389	1.793		
Total	46 (1)	6365.763			

Appendix 5.3.4.3 Propionate production (molar %) following incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Inocula	2	124.496	62.248	33.84	< 0.001
Feed	3	1914.829	638.276	346.99	< 0.001
Inocula.Feed	6	1590.604	265.101	144.12	< 0.001
Replicate	3	4.251	1.417	0.77	0.519
Residual	32 (1)	58.863	1.839		
Total	46 (1)	3681.748			

Appendix 5.3.4.4 Butyrate production (molar %) following incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Inocula	2	213.039	106.520	58.22	< 0.001
Feed	3	556.100	185.367	101.31	< 0.001
Inocula.Feed	6	318.840	53.140	29.04	< 0.001
Replicate	3	5.022	1.674	0.91	0.445
Residual	32 (1)	58.550	1.830		
Total	46 (1)	1150.224			

Appendix 5.3.4.5 Valerate production (molar %) following incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Inocula	2	3.57928	1.78964	40.39	< 0.001
Feed	3	34.96719	11.65573	263.04	< 0.001
Inocula.Feed	6	30.02005	5.00334	112.91	< 0.001
Replicate	3	0.58059	0.19353	4.37	0.011
Residual	32 (1)	1.41796	0.04431		
Total	46 (1)	70.50469			

Appendix 5.3.5 pH following incubation of oatfeed, naked oats, soya hulls and unmolassed sugar beet pulp with either an equine caecal, equine faecal or a bovine rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Inocula	2	0.0385042	0.0192521	167.93	< 0.001
Feed	3	0.2189833	0.0729944	636.69	< 0.001
Inocula.Feed	6	0.0278792	0.0046465	40.53	< 0.001
Replicate	3	0.0006167	0.0002056	1.79	0.168
Residual	33	0.0037833	0.0001146		
Total	47	0.2897667			

APPENDIX 6

Appendix 6.1 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 0.50 g dry matter of fresh, freeze, oven or microwave dried perennial ryegrass (*Lolium perenne*) incubated with a rumen microbial inoculum.

Drying treatment	Source[1]	DF	SS	MS	F-ratio[2]
Freeze versus oven	Between rates (b and c)	2	23.1456	11.5728	5.3299*
	Between gas pool (A and B)	2	102.4278	51.2139	28.5867***
	within subset	24	52.1110	2.1713	
Freeze versus microwave	Between rates (b and c)	2	36.5548	18.2774	7.7969**
	Between gas pool (A and B)	2	111.5955	55.7977	23.8024***
	within subset	24	56.2602	2.3442	
Freeze versus fresh	Between rates (b and c)	2	14.6649	7.3325	10.4601***
	Between gas pool (A and B)	2	3453.8410	1726.9200	2463.5093***
	within subset	24	16.8236	0.7010	
Oven versus microwave	Between rates (b and c)	2	82.5053	41.2527	12.2375***
	Between gas pool (A and B)	2	2.1986	1.0993	0.3261
	within subset	24	80.9030	3.3710	
Oven versus fresh	Between rates (b and c)	2	4070.9750	2035.4880	1177.8081***
	Between gas pool (A and B)	2	9.6596	4.8298	2.7947
	within subset	24	41.4770	1.7282	
Fresh versus microwave	Between rates (b and c)	2	3013.3930	1506.6970	792.6229***
	Between gas pool (A and B)	2	18.6055	9.3028	4.8939*
	within subset	24	45.6212	1.9009	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

***p < 0.001, **p < 0.01 and *p < 0.05. The F values were 9.339, 5.614 and 3.403 for 0.1, 1 and 5 %, respectively (Lindley & Scott, 1990).

Appendix 6.2.1 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	0.0007047	0.0002349	10.45	0.004
Replicate	3	0.0000834	0.0000278	1.24	0.358
Residual	8 (1)	0.0001798	0.0000225		
Total	14 (1)	0.0009419			

Appendix 6.2.2 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	0.037945	0.012648	33.60	< 0.001
Replicate	3	0.001764	0.000588	1.56	0.273
Residual	8 (1)	0.003011	0.000376		
Total	14 (1)	0.039284			

Appendix 6.2.3 The cumulative gas production, A (ml; France *et al.*, 1993), obtained during incubation of freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	2146.705	715.568	112.13	< 0.001
Replicate	3	32.691	10.897	1.71	0.242
Residual	8 (1)	51.053	6.382		
Total	14 (1)	2205.514			

Appendix 6.2.4 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	1082.89	360.96	31.00	< 0.001
Replicate	3	106.22	35.41	3.04	0.093
Residual	8 (1)	93.14	11.64		
Total	14 (1)	1123.71			

Appendix 6.2.5 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	9.50043	3.16681	54.76	< 0.001
Replicate	3	0.28554	0.09518	1.65	0.255
Residual	8 (1)	0.46263	0.05783		
Total	14 (1)	9.09600			

Appendix 6.2.6 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	25.834	8.611	4.57	0.038
Replicate	3	3.151	1.050	0.56	0.658
Residual	8 (1)	15.080	1.885		
Total	14 (1)	37.411			

Appendix 6.2.7 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	366.24	122.08	3.53	0.068
Replicate	3	97.49	32.50	0.94	0.465
Residual	8 (1)	276.32	34.54		
Total	14 (1)	740.05			

Appendix 6.3 Dry matter loss from freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	712.40	237.47	7.58	0.010
Replicate	3	51.32	17.11	0.55	0.665
Residual	8 (1)	250.68	31.33		
Total	14 (1)	1013.11			

Appendix 6.4.1 Total volatile fatty acid (VFA) production (mmol l^{-1}) from freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	84.0412	28.0137	29.72	< 0.001
Replicate	3	9.2568	3.0856	3.27	0.080
Residual	8 (1)	7.5413	0.9427		
Total	14 (1)	96.3336			

Appendix 6.4.2 Acetate production (molar %) from freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	40.5384	13.5128	17.14	< 0.001
Replicate	3	1.9136	0.6379	0.81	0.523
Residual	8 (1)	6.3059	0.7882		
Total	14 (1)	39.7958			

Appendix 6.4.3 Propionate production (molar %) from freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	24.6659	8.2220	13.75	0.002
Replicate	3	1.1947	0.3982	0.67	0.596
Residual	8 (1)	4.7839	0.5980		
Total	14 (1)	25.1392			

Appendix 6.4.4 Butyrate production (molar %) from freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	3.6792	1.2264	5.20	0.028
Replicate	3	1.6692	0.5564	2.36	0.148
Residual	8 (1)	1.8864	0.2358		
Total	14 (1)	6.0670			

Appendix 6.4.5 Valerate production (molar %) from freeze dried, oven dried (60 °C), microwave dried and fresh ryegrass (*Lolium perenne*) after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	0.69376	0.23125	5.18	0.028
Replicate	3	0.42767	0.14256	3.19	0.084
Residual	8 (1)	0.35699	0.04462		
Total	14 (1)	1.42926			

Appendix 6.5 The pH of the culture fluid at 120 h, following the incubation of 0.5 g DM freeze dried, oven dried (60 °C), microwave dried or fresh ryegrass (*Lolium perenne*) using the pressure transducer technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Drying treatment	3	0.012554	0.004185	1.81	0.223
Replicate	3	0.034709	0.011570	5.01	0.030
Residual	8 (1)	0.018489	0.002311		
Total	14 (1)	0.051440			

Appendix 6.6.1 Rhamnose content (mg g^{-1} DM) of different particle sizes of perennial ryegrass hay (*Lolium perenne*).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	7.036	1.759	0.85	0.534
Replicate	2	4.741	2.371	1.14	0.367
Residual	8	16.632	2.079		
Total	14	28.409			

Appendix 6.6.2 Arabinose content (mg g^{-1} DM) of different particle sizes of perennial ryegrass hay (*Lolium perenne*).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	509.620	127.405	61.22	< 0.001
Replicate	2	0.465	0.233	0.11	0.896
Residual	8	16.648	2.081		
Total	14	526.733			

Appendix 6.6.3 Xylose content (mg g^{-1} DM) of different particle sizes of perennial ryegrass hay (*Lolium perenne*).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	675.85	168.96	1.96	0.194
Replicate	2	172.56	86.28	1.00	0.409
Residual	8	689.22	86.15		
Total	14	1537.64			

Appendix 6.6.4 Mannose content (mg g^{-1} DM) of different particle sizes of perennial ryegrass hay (*Lolium perenne*).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	7.367	1.842	1.12	0.412
Replicate	2	3.349	1.675	1.02	0.404
Residual	8	13.177	1.647		
Total	14	23.893			

Appendix 6.6.5 Galactose content (mg g^{-1} DM) of different particle sizes of perennial ryegrass hay (*Lolium perenne*).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	95.707	23.927	18.99	< 0.001
Replicate	2	1.445	0.723	0.57	0.585
Residual	8	10.081	1.260		
Total	14	107.233			

Appendix 6.6.6 Glucose content (mg g^{-1} DM) of different particle sizes of perennial ryegrass hay (*Lolium perenne*).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	3407.7	851.9	2.11	0.172
Replicate	2	672.0	336.0	0.83	0.470
Residual	8	3233.2	404.2		
Total	14	7313.0			

Appendix 6.6.7 Uronic acid content (mg g^{-1} DM) of different particle sizes of perennial ryegrass hay (*Lolium perenne*).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	175.303	43.826	25.63	<0.001
Replicate	2	5.232	2.616	1.53	0.274
Residual	8	13.681	1.710		
Total	14	194.216			

Appendix 6.6.8 Total NSP content (mg g^{-1} DM) of different particle sizes of perennial ryegrass hay (*Lolium perenne*).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	11570.8	2892.7	3.79	0.051
Replicate	2	1341.6	670.8	0.88	0.452
Residual	8	6101.0	762.6		
Total	14	19013.3			

Appendix 6.7 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 0.75 g naked oats of varying particle size incubated with a rumen microbial inoculum.

Particle size	Source[1]	DF	SS	MS	F-ratio[2]
whole versus half	Between rates (b and c)	2	3307.1140	1653.5570	238.58***
	Between gas pool (A and B)	2	174501.2000	87250.5800	12589.00***
	within subset	22	152.4748	6.9307	
whole versus quarter	Between rates (b and c)	2	3754.6970	1877.3480	234.30***
	Between gas pool (A and B)	2	188818.4000	94409.21	11782.59***
	within subset	22	176.2762	8.0126	
whole versus coarse	Between rates (b and c)	2	3724.9690	1862.4840	233.59***
	Between gas pool (A and B)	2	190853.1000	95426.5300	11968.41***
	within subset	22	175.4109	7.9732	
whole versus 1mm	Between rates (b and c)	2	4137.2190	2068.6100	202.33***
	Between gas pool (A and B)	2	197255.4000	98627.7000	9646.59***
	within subset	22	224.9295	10.2241	
half versus quarter	Between rates (b and c)	2	233.0971	116.5486	8.9279**
	Between gas pool (A and B)	2	311.3582	155.6791	11.9254***
	within subset	24	313.3065	13.0544	
half versus coarse	Between rates (b and c)	2	321.8318	160.9159	12.36***
	Between gas pool (A and B)	2	663.2278	331.6139	25.47***
	within subset	24	312.4433	13.0185	
half versus 1mm	Between rates (b and c)	2	1715.1210	857.6058	56.86***
	Between gas pool (A and B)	2	2532.1160	1266.0580	83.95***
	within subset	24	361.9592	15.0816	
	Between rates	2	22.8579	11.4290	0.82

	(b and c)				
quarter versus coarse	Between gas pool (A and B)	2	117.2745	58.6372	4.19*
	within subset	24	336.2358	14.0098	
quarter versus 1mm	Between rates (b and c)	2	852.7007	426.3504	26.53***
	Between gas pool (A and B)	2	1409.7310	704.8656	43.85***
	within subset	24	385.7516	16.0730	
coarse versus 1mm	Between rates (b and c)	2	609.5927	304.7964	19.01***
	Between gas pool (A and B)	2	711.2036	355.6018	22.1731***
	within subset	24	384.8991	16.0375	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
***p < 0.001, **p < 0.01 and *p < 0.05. The F values at the 0.1, 1 and 5 % levels were 9.612 and 9.339, 5.719 and 5.614, and 3.443 and 3.403 for 22 and 24 degrees of freedom respectively (Lindley & Scott, 1990).

Appendix 6.8.1 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	0.0581349	0.0145337	577.99	< 0.001
Replicate	3	0.0000590	0.0000197	0.78	0.528
Residual	11 (1)	0.0002766	0.0000251		
Total	18 (1)	0.0461188			

Appendix 6.8.2 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	0.7947877	0.1986969	340.93	< 0.001
Replicate	3	0.0012735	0.0004245	0.73	0.556
Residual	11 (1)	0.0064109	0.0005828		
Total	18 (1)	0.6293289			

Appendix 6.8.3 The cumulative gas production, A (ml; France *et al.*, 1993), obtained during incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	18410.9	4602.7	43.96	< 0.001
Replicate	3	358.9	119.6	1.14	0.375
Residual	11 (1)	1151.8	104.7		
Total	18 (1)	16734.1			

Appendix 6.8.4 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	1140.18	285.04	3.86	0.034
Replicate	3	716.07	238.69	3.23	0.064
Residual	11 (1)	811.66	73.79		
Total	18 (1)	2250.31			

Appendix 6.8.5 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	15.1349	3.7837	9.43	0.001
Replicate	3	0.8485	0.2828	0.70	0.569
Residual	11 (1)	4.4136	0.4012		
Total	18 (1)	18.2344			

Appendix 6.8.6 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	748.489	187.122	162.48	< 0.001
Replicate	3	4.915	1.638	1.42	0.289
Residual	11 (1)	12.668	1.152		
Total	18 (1)	616.608			

Appendix 6.8.7 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	82615.516	20653.879	0.000037	< 0.001
Replicate	3	8.277	2.759	1.83	0.200
Residual	11 (1)	16.566	1.506		
Total	18 (1)	65139.571			

Appendix 6.9 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 0.75 g hay of varying particle size incubated with a rumen microbial inoculum.

Particle size	Source[1]	DF	SS	MS	F-ratio[2]
2.4 versus 1.2mm	Between rates (b and c)	2	36.9503	18.4751	9.14**
	Between gas pool (A and B)	2	319.5446	159.7723	79.08***
	within subset	24	48.4898	2.0204	
2.4 versus 0.6 mm	Between rates (b and c)	2	256.0573	128.0286	30.68***
	Between gas pool (A and B)	2	5159.8780	2579.9390	618.36***
	within subset	24	100.1324	4.1722	
2.4 versus 0.3 mm	Between rates (b and c)	2	1407.1800	703.5898	72.48***
	Between gas pool (A and B)	2	31499.4700	15749.7400	1622.51***
	within subset	24	232.9669	9.7070	
2.4 versus 0.15 mm	Between rates (b and c)	2	3318.5030	1659.2510	151.32***
	Between gas pool (A and B)	2	70148.9400	35074.4700	3198.62***
	within subset	24	263.1730	10.9655	
1.2 versus 0.6 mm	Between rates (b and c)	2	93.6705	46.8352	9.56***
	Between gas pool (A and B)	2	2951.1170	1475.5590	301.11***
	within subset	24	117.6088	4.9004	
1.2 versus 0.3 mm	Between rates (b and c)	2	973.9058	486.9529	46.66***
	Between gas pool (A and B)	2	25514.1200	12757.0600	1222.41***
	within subset	24	250.4629	10.4360	
1.2 versus 0.15 mm	Between rates (b and c)	2	2667.0320	1333.5160	114.04***
	Between gas pool (A and B)	2	60979.6300	30489.8100	2607.33***
	within subset	24	280.6526	11.6939	
0.6 versus 0.3 mm	Between rates (b and c)	2	531.0347	265.5174	21.09***
	Between gas pool (A and B)	2	11148.2700	5574.1370	442.78***
	within subset	24	302.1365	12.5890	
0.6 versus 0.15 mm	Between rates (b and c)	2	2068.2070	1034.1040	74.68***
	Between gas pool (A and B)	2	37246.6800	18623.3400	1344.98***

	within subset	24	332.3177	13.8466	
0.3 versus 0.15 mm	Between rates (b and c)	2	575.7506	287.8753	14.85***
	Between gas pool (A and B)	2	7661.8690	3830.9340	197.66***
	within subset	24	465.1562	19.3815	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
***p < 0.001, **p < 0.01 and *p < 0.05. The F values were 9.339, 5.614 and 3.403 for 0.1, 1 and 5 %, respectively (Lindley & Scott, 1990).

Appendix 6.10.1 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of different particle sizes of hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	0.00523	0.00131	646.88	< 0.001
Replicate	3	0.00006	0.00002	9.67	0.002
Residual	12	0.00002	0.00000		
Total	19	0.00531			

Appendix 6.10.2 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of different particle sizes of hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	0.0415128	0.0103782	110.27	< 0.001
Replicate	3	0.0005934	0.0001978	2.10	0.153
Residual	12	0.0011294	0.0000941		
Total	19	0.0432357			

Appendix 6.10.3 The cumulative gas production, A (ml; France *et al.*, 1993), obtained during incubation of different particle sizes of hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	23918.89	5979.72	69.18	< 0.001
Replicate	3	51.06	17.02	0.20	0.896
Residual	12	1037.23	86.44		
Total	19	25007.17			

Appendix 6.10.4 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of different particle sizes of hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	11673.26	2918.32	32.59	< 0.001
Replicate	3	75.64	25.21	0.28	0.838
Residual	12	1074.45	89.54		
Total	19	12823.35			

Appendix 6.10.5 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of different particle sizes of hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	0.37093	0.09273	1.57	0.246
Replicate	3	0.17298	0.05766	0.97	0.437
Residual	12	0.71060	0.05922		
Total	19	1.25451			

Appendix 6.10.6 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of different particle sizes of hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	428.7153	107.1788	182.04	< 0.001
Replicate	3	9.5316	3.1772	5.40	0.014
Residual	12	7.0651	0.5888		
Total	19	445.3120			

Appendix 6.10.7 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of different particle sizes of hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	6971.600	1742.900	301.44	< 0.001
Replicate	3	138.663	46.221	7.99	0.003
Residual	12	69.384	5.782		
Total	19	7179.647			

Appendix 6.11 Dry matter loss from different particle sizes of naked oats after a 120 h incubation with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	11323.652	2830.913	350.14	< 0.001
Replicate	3	69.208	23.069	2.85	0.091
Residual	10 (2)	80.850	8.085		
Total	17 (2)	11301.207			

Appendix 6.12 Dry matter loss from different particle sizes of perennial ryegrass hay (*Lolium perenne*) after a 145 h incubation with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	3613.19	903.30	14.24	< 0.001
Replicate	3	310.00	103.33	1.63	0.235
Residual	12	761.02	63.42		
Total	19	4684.22			

Appendix 6.13.1 Total volatile fatty acid (VFA) production (mmol l^{-1}) following a 120 h incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	2805.439	701.360	211.03	< 0.001
Replicate	3	48.796	16.265	4.89	0.019
Residual	12	39.881	3.323		
Total	19	2894.117			

Appendix 6.13.2 Acetate production (molar %) following a 120 h incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	343.602	85.901	58.29	< 0.001
Replicate	3	4.065	1.355	0.92	0.461
Residual	12	17.684	1.474		
Total	19	365.351			

Appendix 6.13.3 Propionate production (molar %) following a 120 h incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	1015.0132	253.7533	723.95	< 0.001
Replicate	3	1.7320	0.5773	1.65	0.231
Residual	12	4.2061	0.3505		
Total	19	1020.9513			

Appendix 6.13.4 Butyrate production (molar %) following a 120 h incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	425.860	106.465	99.79	< 0.001
Replicate	3	1.434	0.478	0.45	0.723
Residual	12	12.803	1.067		
Total	19	440.097			

Appendix 6.13.5 Valerate production (molar %) following a 120 h incubation of different particle sizes of naked oats with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	30.9675	7.7419	9.10	0.001
Replicate	3	2.0092	0.6697	0.79	0.524
Residual	12	10.2141	0.8512		
Total	19	43.1908			

Appendix 6.14.1 Total volatile fatty acid (VFA) production (mmol l^{-1}) following a 145 h incubation of different particle sizes of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f (m.v.)	s.s.	m.s.	v.r.	F pr.
Size	4	247.43	61.86	2.98	0.068
Replicate	3	29.84	9.95	0.48	0.704
Residual	11 (1)	228.60	20.78		
Total	18 (1)	486.16			

Appendix 6.14.2 Acetate production (molar %) following a 145 h incubation of different particle sizes of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	15.720	3.930	2.51	0.102
Replicate	3	7.085	2.362	1.51	0.267
Residual	11 (1)	17.225	1.566		
Total	18 (1)	37.409			

Appendix 6.14.3 Propionate production (molar %) following a 145 h incubation of different particle sizes of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	10.1484	2.5371	5.23	0.013
Replicate	3	3.0195	1.0065	2.08	0.162
Residual	11 (1)	5.3323	0.4848		
Total	18 (1)	17.5300			

Appendix 6.14.4 Butyrate production (molar %) following a 145 h incubation of different particle sizes of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	0.9327	0.2332	0.94	0.476
Replicate	3	0.3871	0.1290	0.52	0.676
Residual	11 (1)	2.7237	0.2476		
Total	18 (1)	3.8669			

Appendix 6.14.5 Valerate production (molar %) following a 145 h incubation of different particle sizes of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Size	4	0.9184	0.2296	1.19	0.369
Replicate	3	0.7822	0.2607	1.35	0.309
Residual	11 (1)	2.1251	0.1932		
Total	18 (1)	3.7801			

Appendix 6.15 pH following a 120 h incubation of different particle sizes of naked oats (*Avena nuda*) with a rumen microbial inoculum.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Size	4	0.0896799	0.0224200	77.31	< 0.001
Replicate	3	0.0064200	0.0021400	7.38	0.005
Residual	12	0.0034800	0.0002900		
Total	19	0.0995798			

Appendix 6.16 pH following a 145 h incubation of different particle sizes of perennial ryegrass hay (*Lolium perenne*) with a rumen microbial inoculum.

Source of variation	d.f (m.v.)	s.s.	m.s.	v.r.	F pr.
Size	4	0.021961	0.005490	2.58	0.102
Replicate	3	0.051120	0.017040	8.02	0.005
Residual	10 (2)	0.021240	0.002124		
Total	17 (2)	0.090050			

APPENDIX 7

Appendix 7.1 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) for each feedstuff fermented using the Menke (MT) or pressure transducer technique (PTT) with a medium to rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1.

Sample	Source[1]	DF	SS	MS	F-ratio[2]
Naked oats 2:1 (M:RF)	Between rates (b and c)	2	134.8530	67.4265	25.01
	Between gas pool (A and B)	2	1083.0830	541.5417	200.86
	within subset	29	78.1856	2.6961	
Naked oats 6:1 (M:RF)	Between rates (b and c)	2	501.2474	250.6237	28.67
	Between gas pool (A and B)	2	361.2156	180.6078	20.66
	within subset	30	262.2330	8.7408	
Naked oats 9:1 (M:RF)	Between rates (b and c)	2	684.6440	342.3220	38.34
	Between gas pool (A and B)	2	399.8087	199.9044	22.39
	within subset	30	267.8406	8.9280	
Oatfeed 2:1 (M:RF)	Between rates (b and c)	2	11.5701	5.7850	9.85
	Between gas pool (A and B)	2	189.5018	94.7509	161.39
	within subset	30	17.6142	0.5871	
Oatfeed 6:1 (M:RF)	Between rates (b and c)	2	22.5143	11.2572	37.65
	Between gas pool (A and B)	2	206.5825	103.2912	345.46
	within subset	30	8.9691	0.2990	
Oatfeed 9:1 (M:RF)	Between rates (b and c)	2	17.5196	8.7598	26.88
	Between gas pool (A and B)	2	427.3919	213.6959	655.71
	within subset	30	9.7759	0.3259	
Ryegrass 2:1 (M:RF)	Between rates (b and c)	2	28.3353	14.1676	10.96
	Between gas pool (A and B)	2	204.9951	102.4975	79.30
	within subset	30	38.7755	1.2925	

Ryegrass 6:1 (M:RF)	Between rates (b and c)	2	114.4367	57.2183	41.52
	Between gas pool (A and B)	2	222.8210	111.4105	80.85
	within subset	30	41.3400	1.3780	
Ryegrass 9:1 (M:RF)	Between rates (b and c)	2	82.6025	41.3013	20.57
	Between gas pool (A and B)	2	129.0163	64.5082	32.12
	within subset	30	60.2479	2.0083	

[1] Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).
[2]F-ratio calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.
The 1% value for F was 5.42 or 5.39, for 29 or 30 within subset degrees of freedom respectively (Lindley & Scott, 1990).

Appendix 7.2.1 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of naked oats, oatfeed and ryegrass with a medium : rumen fluid ratio of either 2:1, 6:1 or 9:1 using the pressure transducer technique and the Menke technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Technique	1	0.046858	0.046858	82.06	<0.001
M:RF	2	0.119158	0.059579	104.34	<0.001
Substrate	2	0.300648	0.150324	263.27	<0.001
Technique.M:RF	2	0.027487	0.013743	24.07	<0.001
Technique.Substrate	2	0.076028	0.038014	66.58	<0.001
M:RF.Substrate	4	0.222978	0.055745	97.63	<0.001
Technique.M:RF:substrate	4	0.048914	0.012228	21.42	<0.001
Replicates	2	0.000937	0.000468	0.82	0.449
Residual	33 (1)	0.018842	0.000571		
Total	52 (1)	0.861831			

Appendix 7.2.2 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of naked oats, oatfeed and ryegrass with a medium : rumen fluid ratio of either 2:1, 6:1 or 9:1 using the pressure transducer technique and the Menke technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Technique	1	0.22644	0.22644	16.81	<0.001
M:RF	2	2.23524	1.11762	82.98	<0.001
Substrate	2	3.45438	1.72719	128.24	<0.001
Technique.M:RF	2	0.33943	0.16971	12.60	<0.001
Technique.Substrate	2	0.77908	0.38954	28.92	<0.001
M:RF.Substrate	4	3.68675	0.92169	68.43	<0.001
Technique.M:RF:substrate	4	0.62688	0.15672	11.64	<0.001
Replicates	2	0.01860	0.00930	0.69	0.508
Residual	33 (1)	0.44445	0.01347		
Total	52 (1)	11.79113			

Appendix 7.2.3 The cumulative gas production, A (ml; France *et al.*, 1993), obtained during incubation of naked oats, oatfeed and ryegrass with a medium : rumen fluid ratio of either 2:1, 6:1 or 9:1 using the pressure transducer technique and the Menke technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Technique	1	948.67	948.67	58.84	<0.001
M:RF	2	105.30	52.65	3.27	0.051
Substrate	2	2978.72	1489.36	92.38	<0.001
Technique.M:RF	2	143.22	71.61	4.44	0.020
Technique.Substrate	2	686.75	343.37	21.30	<0.001
M:RF.Substrate	4	666.76	166.69	10.34	<0.001
Technique.M:RF:substrate	4	98.86	24.71	1.53	0.215
Replicates	2	11.08	5.54	0.34	0.712
Residual	33 (1)	532.01	16.12		
Total	52 (1)	6128.71			

Appendix 7.2.4 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of naked oats, oatfeed and ryegrass with a medium : rumen fluid ratio of either 2:1, 6:1 or 9:1 using the pressure transducer technique and the Menke technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Technique	1	1219.79	1219.79	12.30	0.001
M:RF	2	6260.63	3130.31	31.57	<0.001
Substrate	2	7852.39	3926.19	39.59	<0.001
Technique.M:RF	2	600.84	300.42	3.03	0.062
Technique.Substrate	2	556.75	278.37	2.81	0.075
M:RF.Substrate	4	14027.94	3506.99	35.36	<0.001
Technique.M:RF:substrate	4	1682.96	420.74	4.24	0.007
Replicates	2	27.25	13.62	0.14	0.872
Residual	33 (1)	3272.52	99.17		
Total	52 (1)	35202.24			

Appendix 7.2.5 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of naked oats, oatfeed and ryegrass with a medium : rumen fluid ratio of either 2:1, 6:1 or 9:1 using the pressure transducer technique and the Menke technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Technique	1	0.2015	0.2015	1.58	0.217
M:RF	2	4.6021	2.3011	18.07	<0.001
Substrate	2	16.9372	8.4686	66.49	<0.001
Technique.M:RF	2	0.0557	0.0279	0.22	0.805
Technique.Substrate	2	3.1554	1.5777	12.39	<0.001
M:RF.Substrate	4	7.0660	1.7665	13.87	<0.001
Technique.M:RF:substrate	4	0.4544	0.1136	0.89	0.480
Replicates	2	0.0438	0.0219	0.17	0.843
Residual	33 (1)	4.2032	0.1274		
Total	52 (1)	36.7194			

Appendix 7.2.6 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of naked oats, oatfeed and ryegrass with a medium : rumen fluid ratio of either 2:1, 6:1 or 9:1 using the pressure transducer technique and the Menke technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Technique	1	316.009	316.009	81.23	<0.001
M:RF	2	176.639	88.319	22.70	<0.001
Substrate	2	8888.405	4444.202	1142.39	<0.001
Technique.M:RF	2	39.401	19.700	5.06	0.012
Technique.Substrate	2	28.021	14.010	3.60	0.038
M:RF.Substrate	4	461.714	115.429	29.67	<0.001
Technique.M:RF:substrate	4	105.402	26.351	6.77	<0.001
Replicates	2	3.838	1.919	0.49	0.615
Residual	33 (1)	128.379	3.890		
Total	52 (1)	10045.031			

Appendix 7.2.7 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of naked oats, oatfeed and ryegrass with a medium : rumen fluid ratio of either 2:1, 6:1 or 9:1 using the pressure transducer technique and the Menke technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Technique	1	2615.9	2615.9	24.49	<0.001
M:RF	2	440.4	220.2	2.06	0.143
Substrate	2	156744.4	78372.2	733.84	<0.001
Technique.M:RF	2	1459.2	729.6	6.83	0.003
Technique.Substrate	2	166.4	83.2	0.78	0.467
M:RF.Substrate	4	13376.5	3344.1	31.31	<0.001
Technique.M:RF:substrate	4	3785.2	946.3	8.86	<0.001
Replicates	2	38.6	19.3	0.18	0.835
Residual	33 (1)	3524.3	106.8		
Total	52 (1)	180189.5			

Appendix 7.3.1 The fractional rate of gas production (μ) at 12 h incubation obtained during incubation of naked oats, oatfeed and ryegrass with a medium : rumen fluid ratio of either 2:1, 6:1 or 9:1 using the pressure transducer technique and the Menke technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Technique	1	0.0218157	0.0218157	319.49	<0.001
M:RF	2	0.0167550	0.0083775	122.69	<0.001
Substrate	2	0.0958559	0.0479279	701.90	<0.001
Technique.M:RF	2	0.0066973	0.0033486	49.04	<0.001
Technique.Substrate	2	0.0223514	0.0111757	163.67	<0.001
M:RF.Substrate	4	0.0384871	0.0096218	140.91	<0.001
Technique.M:RF:substrate	4	0.0115207	0.0028802	42.18	<0.001
Replicates	2	0.0001895	0.0000947	1.39	0.264
Residual	33 (1)	0.0022534	0.0000683		
Total	52 (1)	0.2156753			

Appendix 7.3.2 The fractional rate of gas production (μ) at 24 h incubation obtained during incubation of naked oats, oatfeed and ryegrass with a medium : rumen fluid ratio of either 2:1, 6:1 or 9:1 using the pressure transducer technique and the Menke technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Technique	1	0.028194	0.028194	178.84	<0.001
M:RF	2	0.037163	0.018581	117.86	<0.001
Substrate	2	0.141070	0.070535	447.41	<0.001
Technique.M:RF	2	0.011336	0.005668	35.95	<0.001
Technique.Substrate	2	0.034750	0.017375	110.21	<0.001
M:RF.Substrate	4	0.076729	0.019182	121.67	<0.001
Technique.M:RF:substrate	4	0.019793	0.004948	31.39	<0.001
Replicates	2	0.000329	0.000164	1.04	0.364
Residual	33 (1)	0.005202	0.000158		
Total	52 (1)	0.354470			

Appendix 7.3.3 The fractional rate of gas production (μ) at 48 h incubation obtained during incubation of naked oats, oatfeed and ryegrass with a medium : rumen fluid ratio of either 2:1, 6:1 or 9:1 using the pressure transducer technique and the Menke technique.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Technique	1	0.033173	0.033173	132.86	<0.001
M:RF	2	0.056361	0.028180	112.86	<0.001
Substrate	2	0.180366	0.090183	361.19	<0.001
Technique.M:RF	2	0.015335	0.007668	30.71	<0.001
Technique.Substrate	2	0.045161	0.022581	90.44	<0.001
M:RF.Substrate	4	0.111619	0.027905	111.76	<0.001
Technique.M:RF:substrate	4	0.026972	0.006743	27.01	<0.001
Replicates	2	0.000467	0.000233	0.93	0.403
Residual	33 (1)	0.008239	0.000250		
Total	52 (1)	0.477662			

Appendix 7.4 Dry matter loss during the incubation of naked oats (NO), oatfeed (OF) or ryegrass (RG), in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1, using the pressure transducer technique (PTT) or the Menke technique (MT).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Technique	1	15613.3	15613.3	15.78	< 0.001
Feed	2	2012724.6	1006362.3	1017.05	< 0.001
M:RF	2	3703.4	1851.7	1.87	0.173
Technique. Feed	2	25166.3	12583.1	12.72	< 0.001
Technique. M:RF	2	8068.6	4034.3	4.08	0.028
Feed. M:RF	4	2959.0	739.7	0.75	0.568
Technique. Feed. M:RF	4	8731.0	2182.8	2.21	0.095
Residual	27	26716.4	989.5		
Total	44	1570914.4			

Appendix 7.5.1 Analysis of variance for total volatile fatty acid production (mmol l^{-1}) during the incubation of naked oats (NO), oatfeed (OF) or ryegrass (RG), in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1, using the pressure transducer technique (PTT) or the Menke technique (MT).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Technique	1	307.59	307.59	19.61	< 0.001
Feed	2	3517.01	1758.51	112.11	< 0.001
M:RF	2	155.42	77.71	4.95	0.014
Technique. Feed	2	106.39	53.20	3.39	0.047
Technique.M:RF	2	163.77	81.88	5.22	0.011
Feed. M:RF	4	258.41	64.60	4.12	0.009
Technique.Feed.M:RF	4	49.59	12.40	0.79	0.540
Residual	31	486.27	15.69		
Total	48	4780.34			

Appendix 7.5.2 Analysis of variance for acetate production (molar %) from naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer technique (PTT) or the Menke technique (MT).

Source of variation	d.f. (m.v.)	s.s	m.s.	v.r.	F pr.
Technique	1	9.4016	9.4016	18.39	< 0.001
Feed	2	3495.4421	1747.7211	3417.94	< 0.001
M:RF	2	21.2911	10.6455	20.82	< 0.001
Technique.Feed	2	11.3552	5.6776	11.10	< 0.001
Technique. M:RF	2	14.0587	7.0294	13.75	< 0.001
Feed. M:RF	4	94.4744	23.6186	46.19	< 0.001
Technique.Feed.M:RF	4	9.0373	2.2593	4.42	0.006
Residual	31 (5)	15.8515	0.5113		
Total	48 (5)	3569.0383			

Appendix 7.5.3 Analysis of variance for propionate production (molar %) from naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer technique (PTT) or the Menke technique (MT).

Source of variation	d.f. (m.v.)	s.s	m.s.	v.r.	F pr.
Technique	1	14.5052	14.5052	27.57	< 0.001
Feed	2	1768.8407	884.4203	1681.31	< 0.001
M:RF	2	412.5751	206.2875	392.16	< 0.001
Technique.Feed	2	4.6852	2.3426	4.45	0.020
Technique. M:RF	2	3.6203	1.8102	3.44	0.045
Feed. M:RF	4	553.5551	138.3888	263.08	< 0.001
Technique.Feed.M:RF	4	0.8299	0.2075	0.39	0.811
Residual	31 (5)	16.3070	0.5260		
Total	48 (5)	2699.6174			

Appendix 7.5.4 Analysis of variance for butyrate production (molar %) from naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer technique (PTT) or the Menke technique (MT).

Source of variation	d.f. (m.v.)	s.s	m.s.	v.r.	F pr.
Technique	1	1.8835	1.8835	12.70	0.001
Feed	2	200.3216	100.1608	675.35	< 0.001
M:RF	2	166.9072	83.4536	562.70	< 0.001
Technique.Feed	2	0.5435	0.2717	1.83	0.177
Technique. M:RF	2	0.4890	0.2445	1.65	0.209
Feed. M:RF	4	166.5118	41.6279	280.68	< 0.001
Technique.Feed.M:RF	4	4.5478	1.1369	7.67	< 0.001
Residual	31 (5)	4.5976	0.1483		
Total	48 (5)	486.1018			

Appendix 7.5.5 Analysis of variance for valerate production (molar %) from naked oats (NO), oatfeed (OF) and ryegrass (RG) incubated in a medium : rumen fluid ratio (M:RF) of 2:1, 6:1 or 9:1 using either the pressure transducer technique (PTT) or the Menke technique (MT).

Source of variation	d.f. (m.v.)	s.s	m.s.	v.r.	F pr.
Technique	1	0.27023	0.27023	5.65	0.024
Feed	2	27.61257	13.80628	288.57	< 0.001
M:RF	2	8.29001	4.14500	86.64	< 0.001
Technique.Feed	2	0.25455	0.12727	2.66	0.086
Technique. M:RF	2	1.24271	0.62135	12.99	< 0.001
Feed. M:RF	4	3.68618	0.92155	19.26	< 0.001
Technique.Feed.M:RF	4	0.37243	0.09311	1.95	0.128
Residual	31 (5)	1.48317	0.04784		
Total	48 (5)	41.26680			

APPENDIX 8

Appendix 8.1.1 Chemical composition of the sixteen feedstuffs used to evaluate the potential of the *in vitro* gas production technique for estimating the *in vivo* digestibility and digestible energy content of equine feeds

Feed	DM ($g\ kg^{-1}$)	g kg^{-1} DM					GE ($MJ\ kg^{-1}$ DM)
		ADF	NDF	OM	CP	Starch	
L1	914.0	346.5	424.3	914.1	148.5	0	17.4
L2	879.5	307.2	349.4	901.7	182.4	0	18.3
L3	863.6	287.4		883.6	199.6	0	18.7
OS	913.0	508.8	762.3	949.9	37.0	0	17.3
WS	932.0	506.4		951.0		0	17.7
GH	681.0	396.9	655.5	935.5	97.6	0	19.5
SBM	883.0	72.7	79.3	925.7	519.2	0	19.9
BG	866.0	76.7	207.3	977.6	127.0	597.8	18.4
SB1	868.0	83.9	145.1	959.7	246.0	438.1	18.9
SB2	969.0	71.8	103.5	940.6	382.0	211.6	19.2
NO	879.0	42.0	137.0	982.6	181.3	578.0	18.9
BO	858.0	152.4	305.6	981.0	96.1	406.1	18.7
RNO	871.0	38.5	77.6	982.5	176.4	630.5	19.3
AM	805.0	434.4	610.0	934.7	128.5	170.3	19.3
SH	894.6	457.3	635.7	949.8	120.8	0	18.7
OF	893.3	307.5	599.9	973.3	63.7	185.5	19.4

See section 8.1.2 for details of abbreviations. The starch content of the feedstuffs was determined using the method of McCleary *et al.* (1997).

Appendix 8.2.1.1 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 1.00 g naked oats incubated with a rumen microbial inoculum and avoparcin at concentrations of 0, 1, 10 or 20 ppm.

Avoparcin concentration (ppm)	Source[1]	DF	SS	MS	F-ratio[2]
0 versus 1	Between rates (b and c)	2	479.7261	239.8630	1.904
	Between gas pool (A and B)	2	144.9658	72.4829	0.575
	within subset	42	5290.4690	125.9635	
0 versus 10	Between rates (b and c)	2	307.46939	153.7319	1.501
	Between gas pool (A and B)	2	118.5796	59.2898	0.579
	within subset	46	4710.2820	102.3974	
0 versus 20	Between rates (b and c)	2	14.6289	7.3145	0.069
	Between gas pool (A and B)	2	617.9961	308.9980	2.909
	within subset	45	4780.6310	106.2362	
1 versus 10	Between rates (b and c)	2	499.1069	249.5535	2.346
	Between gas pool (A and B)	2	415.1860	207.5930	1.952
	within subset	44	4680.2610	106.3696	
1 versus 20	Between rates (b and c)	2	597.9121	298.9561	2.706
	Between gas pool (A and B)	2	1211.2620	605.6311	5.482**
	within subset	43	4750.6150	110.4794	
10 versus 20	Between rates (b and c)	2	1294.5980	647.2988	0.629
	Between gas pool (A and B)	2	3381.4060	1690.7030	1.644
	within subset	46	47305.610	1028.3830	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

** $p < 0.01$. The F value (for ≥ 40 df) at the 5, 1 and 0.1 % levels was 3.232, 5.179 and 8.251, respectively.

Appendix 8.2.1.2 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 1.00 g naked oats incubated with a rumen microbial inoculum and penicillin G at concentrations of 0, 1, 10 or 20 ppm.

Penicillin G concentration (ppm)	Source[1]	DF	SS	MS	F-ratio[2]
0 versus 1	Between rates (b and c)	2	1052.4250	526.2125	5.348**
	Between gas pool (A and B)	2	1147.6900	573.8452	5.832**
	within subset	40	3935.9440	98.3986	
0 versus 10	Between rates (b and c)	2	9047.2660	4523.6330	60.454***
	Between gas pool (A and B)	2	10154.430	5077.2160	67.852***
	within subset	44	3292.4080	74.8275	
0 versus 20	Between rates (b and c)	2	11081.370	5540.6860	73.447***
	Between gas pool (A and B)	2	10463.370	5231.6840	69.351***
	within subset	44	3319.2770	75.4381	
1 versus 10	Between rates (b and c)	2	3916.9390	1958.4690	41.061***
	Between gas pool (A and B)	2	4980.7320	2490.3660	52.213***
	within subset	40	1907.8630	47.6966	
1 versus 20	Between rates (b and c)	2	5233.0250	2616.5130	54.096***
	Between gas pool (A and B)	2	4878.4800	2439.2400	50.431***
	within subset	40	1934.7040	48.3676	
10 versus 20	Between rates (b and c)	2	150.4222	75.2111	2.563
	Between gas pool (A and B)	2	162.2903	81.1451	2.765
	within subset	44	1291.1770	29.3449	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

** $p < 0.01$, *** $p < 0.001$. The F value (for ≥ 40 df) at the 5, 1 and 0.1 % levels was 3.232, 5.179 and 8.251, respectively.

Appendix 8.2.1.3 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 1.00 g naked oats incubated with a rumen microbial inoculum and avoparcin (A) or penicillin G (P) at concentrations of 1, 10 or 20 ppm.

Antibiotic concentration (ppm)	Source[1]	DF	SS	MS	F-ratio[2]
1A versus 1P	Between rates (b and c)	2	361.8953	180.9476	1.760
	Between gas pool (A and B)	2	1552.7390	776.3696	7.553**
	within subset	38	3905.9190	102.7873	
1A versus 10P	Between rates (b and c)	2	7565.4250	3782.7130	48.699***
	Between gas pool (A and B)	2	12292.600	6146.3010	79.127***
	within subset	42	3262.3900	77.6760	
1A versus 20P	Between rates (b and c)	2	9472.7290	4736.3640	60.478***
	Between gas pool (A and B)	2	12395.840	6197.9200	79.141***
	within subset	42	3289.2340	78.3151	
10A versus 1P	Between rates (b and c)	2	416.7515	208.3757	2.631
	Between gas pool (A and B)	2	582.6733	291.3367	3.679*
	within subset	42	3325.7390	79.1843	
10A versus 10P	Between rates (b and c)	2	6227.5130	3113.7570	53.401***
	Between gas pool (A and B)	2	8951.0660	4475.5330	76.756***
	within subset	46	2682.2060	58.3088	
10A versus 20P	Between rates (b and c)	2	8029.9720	4014.9860	68.175***
	Between gas pool (A and B)	2	9106.0720	4553.0360	77.311***
	within subset	46	2709.0480	58.8923	
20A versus 1P	Between rates (b and c)	2	1168.6790	584.3396	7.054**
	Between gas pool (A and B)	2	451.1675	225.5837	2.723
	within subset	41	3396.0960	82.8316	
20A versus 10P	Between rates (b and c)	2	8393.4800	4196.7400	68.610***
	Between gas pool (A and B)	2	5908.7190	2954.3590	48.299***
	within subset	45	2752.5490	61.1678	
20A versus 20P	Between rates (b and c)	2	10148.620	5074.3120	82.156***
	Between gas pool	2	6310.6120	3155.3060	51.086***

	(A and B)				
	within subset	45	2779.4000	61.7645	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The F value (for ≥ 40 df) at the 5, 1 and 0.1 % levels was 3.232, 5.179 and 8.251, respectively. Whilst at 38 df, F was 3.245, 5.211 and 8.331 at the 5, 1 and 0.1 % levels, respectively.

Appendix 8.2.1.4 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	0.083677	0.013946	13.53	<0.001
Replicate	2	0.001303	0.000651	0.63	0.550
Residual	11 (1)	0.011336	0.001031		
Total	19 (1)	0.089993			

Appendix 8.2.1.5 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	0.64179	0.10696	7.09	0.003
Replicate	2	0.02575	0.01287	0.85	0.453
Residual	11 (1)	0.16602	0.01509		
Total	19 (1)	0.80417			

Appendix 8.2.1.6 The cumulative gas production, A (ml; France *et al.*, 1993) obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	6085.10	1014.18	14.74	<0.001
Replicate	2	114.04	57.02	0.83	0.462
Residual	11 (1)	756.97	68.82		
Total	19 (1)	6145.93			

Appendix 8.2.1.7 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	22213.9	3702.3	7.53	0.002
Replicate	2	490.4	245.2	0.50	0.621
Residual	11 (1)	5410.2	491.8		
Total	19 (1)	27074.1			

Appendix 8.2.1.8 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	0.73981	0.12330	2.29	0.111
Replicate	2	0.17933	0.08966	1.66	0.234
Residual	11 (1)	0.59265	0.05388		
Total	19 (1)	1.36724			

Appendix 8.2.1.9 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	96.08244	16.01374	177.27	<0.001
Replicate	2	0.20290	0.10145	1.12	0.360
Residual	11 (1)	0.99369	0.09034		
Total	19 (1)	83.52786			

Appendix 8.2.1.10 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f (m.v)	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	1310.871	218.479	70.76	<0.001
Replicate	2	1.506	0.753	0.24	0.788
Residual	11 (1)	33.964	3.088		
Total	19 (1)	1202.162			

Appendix 8.2.2.1 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 1.00 g naked oats incubated with a rumen microbial inoculum and monensin at concentrations of 0, 1, 10 or 20 ppm.

Monensin concentration (ppm)	Source[1]	DF	SS	MS	F-ratio[2]
0 versus 1	Between rates (b and c)	2	155.2427	77.6213	0.583
	Between gas pool (A and B)	2	152.4712	76.2356	0.573
	within subset	47	6254.8650	133.0822	
0 versus 10	Between rates (b and c)	2	492.5239	246.2620	2.046
	Between gas pool (A and B)	2	2196.0950	1098.0470	9.124***
	within subset	45	5415.3380	120.3409	
0 versus 20	Between rates (b and c)	2	268.4409	134.2205	1.087
	Between gas pool (A and B)	2	4884.3790	2442.1890	19.786***
	within subset	44	5430.7440	123.4260	
1 versus 10	Between rates (b and c)	2	1403.1100	701.5552	6.18**
	Between gas pool (A and B)	2	4334.3800	2167.1900	19.10***
	within subset	45	5105.0360	113.4452	
1 versus 20	Between rates (b and c)	2	58.4404	29.2202	0.270
	Between gas pool (A and B)	2	5274.8430	2637.4210	24.369***
	within subset	43	4653.7220	108.2261	
10 versus 20	Between rates (b and c)	2	88.7744	44.3872	0.437
	Between gas pool (A and B)	2	423.8101	211.9050	2.085
	within subset	41	4167.8060	101.6538	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

** $p < 0.01$, *** $p < 0.001$. The F value (for ≥ 40 df) at the 5, 1 and 0.1 % levels was 3.232, 5.179 and 8.251, respectively.

Appendix 8.2.2.2 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 1.00 g naked oats incubated with a rumen microbial inoculum and chloramphenicol at concentrations of 0, 1, 10 or 20 ppm.

Chloramphenicol concentration (ppm)	Source[1]	DF	SS	MS	F-ratio[2]
0 versus 1	Between rates (b and c)	2	162.6191	81.3096	0.570
	Between gas pool (A and B)	2	1566.4980	783.2488	5.494**
	within subset	42	5987.1060	142.5502	
0 versus 10	Between rates (b and c)	2	316.6016	158.3008	1.069
	Between gas pool (A and B)	2	411.5908	205.795	1.390
	within subset	46	6808.5000	148.0109	
0 versus 20	Between rates (b and c)	2	542.4180	271.2090	1.857
	Between gas pool (A and B)	2	267.6069	133.8035	0.916
	within subset	44	6427.2570	146.0740	
1 versus 10	Between rates (b and c)	2	268.0776	134.0388	0.930
	Between gas pool (A and B)	2	3283.9410	1641.9710	11.394***
	within subset	40	5764.1140	144.1028	
1 versus 20	Between rates (b and c)	2	81.5869	40.7935	0.288
	Between gas pool (A and B)	2	710.6919	355.3459	2.509
	within subset	38	5382.4870	141.6444	
10 versus 20	Between rates (b and c)	2	503.9697	251.9849	1.706
	Between gas pool (A and B)	2	1237.6700	618.8350	4.189*
	within subset	42	6203.8850	147.7115	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The F value (for ≥ 40 df) at the 5, 1 and 0.1 % levels was 3.232, 5.179 and 8.251, respectively. Whilst at 38 df, F was 3.245, 5.211 and 8.331 at the 5, 1 and 0.1 % levels, respectively.

Appendix 8.2.2.3 Parallel curve analysis of the fitted parameters of gas production (France *et al.*, 1993) from 1.00 g naked oats incubated with a rumen microbial inoculum and monensin (M) or chloramphenicol (C) at concentrations of 1, 10 or 20 ppm.

Antibiotic concentration (ppm)	Source[1]	DF	SS	MS	F-ratio[2]
1M versus 1C	Between rates (b and c)	2	524.7856	262.3928	2.065
	Between gas pool (A and B)	2	2177.0470	1088.5240	8.566***
	within subset	41	5210.0840	127.0752	
1M versus 10C	Between rates (b and c)	2	466.6113	233.3057	1.741
	Between gas pool (A and B)	2	195.5542	97.7771	0.729
	within subset	45	6031.4750	134.0328	
1M versus 20C	Between rates (b and c)	2	1131.1050	565.5527	4.304*
	Between gas pool (A and B)	2	752.5576	376.2788	2.864
	within subset	43	5650.2430	131.4010	
10M versus 1C	Between rates (b and c)	2	755.7056	377.8528	3.372*
	Between gas pool (A and B)	2	391.5093	195.7546	1.747
	within subset	39	4370.5690	112.0659	
10M versus 10C	Between rates (b and c)	2	270.2422	135.1211	1.119
	Between gas pool (A and B)	2	3584.8230	1792.4110	14.845***
	within subset	43	5191.9580	120.7432	
10M versus 20C	Between rates (b and c)	2	1245.3700	622.6848	5.307**
	Between gas pool (A and B)	2	1520.2940	760.1472	6.478**
	within subset	41	4810.7180	117.3346	
20M versus 1C	Between rates (b and c)	2	590.0210	295.0105	2.556
	Between gas pool (A and B)	2	1167.9400	583.9702	5.059**
	within subset	38	4385.9690	115.4202	
20M versus 10C	Between rates (b and c)	2	304.9502	152.4751	1.230
	Between gas pool (A and B)	2	6992.9030	3496.4520	28.201***
	within subset	42	5207.3600	123.9848	
	Between rates (b and c)	2	1111.3880	555.6941	4.606*

20M versus 20C	Between gas pool (A and B)	2	3517.4570	1758.7280	14.577***
	within subset	40	4826.1180	120.6530	

[1]Between rates represents the two rate constants b and c, where b = -lnQ and c = -lnZ, and between gas pool represents the parameters A and B in the equation of France *et al.* (1993).

[2]F-ratio was calculated by dividing the mean sum of squares for each variation component by the (within) mean square error.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The F value (for ≥ 40 df) at the 5, 1 and 0.1 % levels was 3.232, 5.179 and 8.251, respectively. Whilst at 38 and 39 df, F was 3.245, 5.211 and 8.331 at the 5, 1 and 0.1 % levels, respectively.

Appendix 8.2.2.4 The rate of gas production, b (h^{-1}; France *et al.*, 1993), obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	0.0132003	0.0022000	7.42	0.002
Replicate	2	0.0033645	0.0016823	5.67	0.018
Residual	12	0.0035581	0.0002965		
Total	20	0.0201229			

Appendix 8.2.2.5 The rate of gas production, c ($h^{-0.5}$; France *et al.*, 1993), obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	0.236160	0.039360	6.56	0.003
Replicate	2	0.045236	0.022618	3.77	0.054
Residual	12	0.071968	0.005997		
Total	20	0.353365			

Appendix 8.2.2.6 The cumulative gas production, A (ml; France *et al.*, 1993) obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	6131.8	1022.0	4.71	0.011
Replicate	2	453.5	226.7	1.04	0.382
Residual	12	2604.9	217.1		
Total	20	9190.3			

Appendix 8.2.2.7 The gas production parameter, B, obtained from the France *et al.* (1993) model during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	6687.5	1114.6	4.03	0.019
Replicate	2	1570.6	785.3	2.84	0.098
Residual	12	3318.0	276.5		
Total	20	11576.1			

Appendix 8.2.2.8 The lag time, L_T (h; France *et al.*, 1993) obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	0.98291	0.16382	6.14	0.004
Replicate	2	0.06123	0.03062	1.15	0.350
Residual	12	0.32040	0.02670		
Total	20	1.36454			

Appendix 8.2.2.9 The time taken to produce 50 % of the total gas production, t_{50} (h; France *et al.*, 1993) obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	1.5353	0.2559	2.40	0.093
Replicate	2	0.3217	0.1608	1.51	0.260
Residual	12	1.2799	0.1067		
Total	20	3.1369			

Appendix 8.2.2.10 The time taken to produce 95 % of the total gas production, t_{95} (h; France *et al.*, 1993) obtained during incubation of 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Antibiotic treatment	6	18.1671	3.0278	6.85	0.002
Replicate	2	5.5118	2.7559	6.23	0.014
Residual	12	5.3053	0.4421		
Total	20	28.9842			

Appendix 8.2.3 Dry matter loss (mg g^{-1}) from 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	10964.5	1827.4	4.30	0.015
Replicates	2	1766.1	883.0	2.08	0.168
Residual	12	5099.2	424.9		
Total	20	17829.8			

Appendix 8.2.4 Dry matter loss (mg g^{-1}) from 1.00 g DM naked oats (*Avena nuda*) incubated with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	1127.5	187.9	1.46	0.272
Replicates	2	321.0	160.5	1.25	0.322
Residual	12	1545.7	128.8		
Total	20	2994.2			

Appendix 8.2.5.1 Total volatile fatty acid (VFA) production (mmol l^{-1}) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f. (m.v)	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	254.82	42.47	3.95	0.027
Replicates	2	14.15	7.08	0.66	0.539
Residual	10 (2)	107.42	10.74		
Total	18 (2)	359.89			

Appendix 8.2.5.2 Acetate production (molar %) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f. (m.v)	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	798.4294	133.0716	324.21	<0.001
Replicates	2	0.2973	0.1486	0.36	0.705
Residual	10 (2)	4.1045	0.4104		
Total	18 (2)	635.2081			

Appendix 8.2.5.3 Propionate production (molar %) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f. (m.v)	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	980.090	163.348	140.47	<0.001
Replicates	2	0.383	0.192	0.16	0.850
Residual	10 (2)	11.629	1.163		
Total	18 (2)	776.764			

Appendix 8.2.5.4 Butyrate production (molar %) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f. (m.v)	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	199.262	33.210	5.08	0.012
Replicates	2	27.972	13.986	2.14	0.169
Residual	10 (2)	65.435	6.543		
Total	18 (2)	286.712			

Appendix 8.2.5.5 Valerate production (molar %) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f. (m.v)	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	14.1298	2.3550	3.94	0.028
Replicates	2	0.2045	0.1023	0.17	0.845
Residual	10 (2)	5.9836	0.5984		
Total	18 (2)	16.1424			

Appendix 8.2.6.1 Total volatile fatty acid (VFA) production (mmol l^{-1}) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	181.224	30.204	4.04	0.019
Replicates	2	74.183	37.092	4.96	0.027
Residual	12	89.736	7.478		
Total	20	345.143			

Appendix 8.2.6.2 Acetate production (molar %) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	11.197	1.866	1.16	0.388
Replicates	2	0.073	0.036	0.02	0.978
Residual	12	19.300	1.608		
Total	20	30.570			

Appendix 8.2.6.3 Propionate production (molar %) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	189.817	31.636	26.47	<0.001
Replicates	2	0.656	0.328	0.27	0.765
Residual	12	14.343	1.195		
Total	20	204.816			

Appendix 8.2.6.4 Butyrate production (molar %) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	245.166	40.861	4.56	0.012
Replicates	2	19.908	9.954	1.11	0.361
Residual	12	107.519	8.960		
Total	20	372.593			

Appendix 8.2.6.5 Valerate production (molar %) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	2.54041	0.42340	10.39	<0.001
Replicates	2	0.09549	0.04775	1.17	0.343
Residual	12	0.48883	0.04074		
Total	20	3.12474			

Appendix 8.2.7.1 L - Lactic acid concentration (μg ml^{-1}) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	1949.84	324.97	6.17	0.004
Replicates	2	126.34	63.17	1.20	0.335
Residual	12	631.64	52.64		
Total	20	2707.82			

Appendix 8.2.7.2 D - Lactic acid concentration (μg ml^{-1}) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	1749.82	291.64	7.83	0.001
Replicates	2	25.03	12.52	0.34	0.721
Residual	12	446.67	37.22		
Total	20	2221.53			

Appendix 8.2.7.3 Total lactic acid concentration (μg ml^{-1}) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	6904.2	1150.7	8.35	0.001
Replicates	2	251.6	125.8	0.91	0.428
Residual	12	1654.3	137.9		
Total	20	8810.2			

Appendix 8.2.8.1 L - Lactic acid concentration (μg ml^{-1}) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f. (m.v)	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	3446.25	574.37	24.05	<0.001
Replicates	2	18.98	9.49	0.40	0.682
Residual	10 (2)	238.80	23.88		
Total	18 (2)	2796.44			

Appendix 8.2.8.2 D - Lactic acid concentration (μg ml^{-1}) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f. (m.v)	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	1686.79	281.13	12.78	<0.001
Replicates	2	75.38	37.69	1.71	0.229
Residual	10 (2)	219.94	21.99		
Total	18 (2)	1522.20			

Appendix 8.2.8.3 Total lactic acid concentration (μg ml^{-1}) from 1.00 g DM naked oats (*Avena nuda*) during incubation with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f. (m.v)	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	2678.05	446.34	7.01	0.004
Replicates	2	162.27	81.14	1.27	0.322
Residual	10 (2)	637.02	63.70		
Total	18 (2)	2742.33			

Appendix 8.2.9 pH following incubation of 1.00 g DM naked oats (*Avena nuda*) with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of avoparcin or penicillin G.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	0.11283	0.01880	1.32	0.321
Replicates	2	0.02435	0.01218	0.85	0.450
Residual	12	0.17111	0.01426		
Total	20	0.30830			

Appendix 8.2.10 pH following incubation of 1.00 g DM naked oats (*Avena nuda*) with a rumen microbial inoculum and 0, 1, 10 or 20 ppm of monensin or chloramphenicol.

Source of variation	d.f.	s.s	m.s	v.r	F pr.
Antibiotic treatment	6	0.099162	0.016527	55.83	<0.001
Replicates	2	0.000181	0.000090	0.31	0.742
Residual	12	0.003552	0.000296		
Total	20	0.102895			

APPENDIX 9 - Publications to date from the work reported in this thesis.

Lowman, R.S., Brooks, A.E., Theodorou, M.K., Dhanoa, M.S & Cuddeford, D (1997) The effect of head-space pressure on gas production profiles as determined using the pressure transducer technique. *In: In Vitro Techniques for Measuring Nutrient Supply to Ruminants, An International Symposium, Reading University, 8 - 10 July 1997.* p 42 (abstract).

Lowman, R.S., Jessop, N.S., Theodorou, M.K., Hererro, M. & Cuddeford, D (1997) A comparison between two *in vitro* gas production techniques to study fermentation profiles of three feedstuffs. *In: In Vitro Techniques for Measuring Nutrient Supply to Ruminants, An International Symposium, Reading University, 8 - 10 July 1997.* p 43 (abstract).

Lowman, R.S., McLean, B.M.L., Theodorou, M.K. & Cuddeford, D (1996) The effect of YEA-SACC^{1026} on the degradation of two fibre sources by rumen inocula *in vitro*, measured using the pressure transducer technique. *Book of Abstracts of the 47th Annual Meeting of the European Association for Animal Production; Lillehammer, Norway, 25 - 29 August 1996,* p 84 (abstract).

Lowman, R.S., Theodorou, M.K., Dhanoa, M.S., Hyslop, J.J & Cuddeford, D (1997) Evaluation of an *in vitro* gas production technique for estimating the *in vivo* digestibility of equine feeds. *Proceedings of the 15th Equine Nutrition and Physiology Symposium, Fort Worth, Texas May 28 - 31, 1997,* pp 1 - 2.

Lowman, R.S., Theodorou, M.K., Hyslop, J.J., Dhanoa, M.S. & Cuddeford, D. (1998) Evaluation of an *in vitro* batch culture technique for estimating the *in vivo* digestibility and digestible energy content of equine feeds using equine faeces as the source of microbial inoculum. Submitted to *Animal Feed Science and Technology.*

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Evaluation of an in vitro batch culture technique for estimating the in vivo digestibility and digestible energy content of equine feeds using equine faeces as the source of microbial inoculum

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Abstract

The suitability of an in vitro batch culture technique for estimating both the dry matter digestibility in vivo (DMIV) and the digestible energy (DE) content of equine feeds was examined. Sixteen feedstuffs of known DMIV and DE contents in equines were incubated at 39 °C with an inoculum prepared from fresh pony faeces. Gas production was recorded throughout using the manual pressure transducer technique. Volatile fatty acid (VFA) concentrations along with dry matter loss (DML) were determined at the end of the incubation period. Prediction equations were developed using cumulative gas production volumes, modelled gas production parameters, values for VFA molar proportions and DML. The best equation for predicting DMIV was obtained using the total VFA production from the feed (TVFA) and the acetic acid (Ac) molar proportion, ie: DMIV = 15431 - 53.58 Ac + 0.04426 Ac^2 + 368.1 log TVFA (R^2 = 0.859; RSD = 51.2). Using only gas production data as predictors yielded the equation DMIV = 234.2 + 4.907 g44 - 8.15 g23 + 13.63 g11 (R^2 = 0.836; RSD = 55.3) where g44, g23 and g11 are cumulative gas volumes measured at 44.5, 23 and 11 hours of incubation. TVFA and Ac were also good predictors of the DE content of the feedstuffs: DE = 251.4 - 0.857 Ac + 0.000692 Ac^2 + 6.55log TVFA (R^2 = 0.865; RSD = 1.04). However, this equation tended to underpredict DE relative to the actual values with a bias of - 0.36 MJ kg^{-1} DM. Using only the modelled gas production parameters Z and T along with DML resulted in a better equation with negligble bias; DE = -0.68 + 0.01087 DML + 6.82 Z - 2.297 log T (R^2 = 0.878; RSD = 0.99). The results demonstrate that equine faeces is a suitable source of microbial inoculum for in vitro gas production studies and that the in vitro batch culture

technique evaluated has considerable potential as a routine predictor of the nutritive value of a wide range of equine feedstuffs.

Key words; equine, gas production, in vitro, prediction, in vivo.

Introduction

Characterisation of feed digestion and routine prediction of nutritive value are essential steps in formulating diets for equines. The use of in vitro digestion techniques developed for use in ruminants (Hershberger et al., 1959; Tilley & Terry, 1963) to investigate forage digestibility by equines has been reported by both Applegate & Hershberger (1969) and Trevor - Jones et al. (1991). Although only a small number of feedstuffs were investigated, ie: alfalfa, timothy and orchard grass; the DM digestibility recorded in vitro was similar to that determined in vivo (R^2 = 0.84; Applegate & Hershberger, 1969). These reported results suggest that ruminant in vitro techniques can be applied to predict the in vivo apparent digestibility of equine feeds.

In ruminant nutrition, in vitro techniques have progressed from end point digestibility measurements (e.g. Tilley & Terry, 1963) to the determination of the kinetics of digestion associated with the fermentation of feedstuffs. This has been achieved by using simple gas measurement techniques such as the pressure transducer method to determine the rate

and extent of gas production during fermentation of particular feeds in batch culture (Theodorou et al., 1994). In these techniques the microbial inoculum employed is prepared from rumen digesta, necessitating the use of surgically modified (fistulated) animals. However gut micro-organisms which are closely associated with plant debris in the rumen are also excreted with plant residues in the faeces (Van Soest, 1982; Theodorou et al., 1993). Faecal material remains largely anaerobic after voiding and the microflora can be viable for several hours after excretion from the digestive tract (Holter, 1991). Thus, faeces may provide an alternative source of inoculum for in vitro digestibility studies. Faeces from sheep and cattle have successfully replaced rumen inocula for in vitro studies of feed digestibility (El Shaer et al., 1987; Akhter et al., 1994; Harris et al., 1995). Equine faeces should also be a readily available source of viable micro-organisms for use in vitro; particularly as equids are hindgut fermenters with little or no post fermentative digestion and absorption of microbial cells as is the case with ruminants.

The first objective of this study was to examine the suitability of an in vitro batch culture technique with an inoculum prepared from equine faeces to obtain values associated with the kinetics of equine feed degradation. The second objective was to undertake a comprehensive evaluation of all the measured values and detail their potential as predictors of the apparent digestibility in vivo and digestible energy content of equine feeds.

Materials and Methods

Feedstuffs

Sixteen feedstuffs were used in the study, ie: three samples of short-chop, dehydrated lucerne (L1, L2, L3), oat straw (OS), wheat straw (WS), grass haylage (GH), soyabean meal (SBM), barley grain (BG), two SBM / BG mixes [33:66 SBM / BG (SB1) and 66:33 SBM / BG (SB2)], naked oats (NO), bruised husked oats (BO), rolled naked oats (RNO), alfalfa / maize mix (AM), soya husks (SH) and a naked oat / oatfeed mix (OF). Dry matter (DM) determinations were carried out using a forced-draught oven at 60 °C for 48 h. Organic matter (OM), crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and gross energy (GE) contents of the feedstuffs were determined according to the methods of the Association of Official Analytical Chemists (1990). The starch content of the feedstuffs was determined using the method of McCleary et al. (1997). The mean, minimum and maximum values along with their standard deviations (SD) for the chemical composition of the sixteen feedstuffs are shown in Table 1. Values observed are consistent with published data for the feedstuffs under study (MAFF, 1990). Apparent DM digestibility in vivo (DMIV) and digestible energy content (DE) of these sixteen feedstuffs were determined previously in mature pony geldings in a series of changeover design experiments (D. Cuddeford & A. Pearson - personal communication). These results are also shown in Table 1.

Table 1 here.

114 Preparation of microbial inoculum

115 Fresh faeces were collected within 1 hour of voiding from four ponies which had ad
116 libitum access to grass hay and water. The microbial inoculum was prepared as described
117 by Theodorou et al. (1994). This involved macerating faeces in a Kenwood liquidiser
118 (Kenwood BL300; Kenwood Ltd., Harant, Hants, UK) followed by straining through three
119 layers of muslin. The liquidiser and collection vessels were continually flushed with
120 carbon dioxide gas to maintain anaerobic conditions throughout the extraction procedure.
121 This process was repeated until 750 ml of microbial inoculum had been collected.

122

123 Preparation of incubation bottles

124 Feedstuffs (750 mg), ground to pass through a 1mm dry mesh screen, were incubated with
125 85 ml of culture medium and 4 ml of freshly prepared reducing agent in 125 ml serum
126 bottles as described by Theodorou and Brooks (1990). Bottles (3 replicates per feedstuff)
127 were then sealed with butyl rubber stoppers and aluminium crimp seals. Anaerobic
128 techniques were employed throughout. Sealed bottles were chilled to 4°C (for not longer
129 than 10 h prior to inoculation), warmed to 39°C and then inoculated with 10 ml of freshly
130 prepared microbial inoculum using a 10 ml syringe fitted with a 23 gauge x 1.5 inch
131 needle. Bottles were incubated at 39°C until the end of the 140 hour fermentation period.

132

133 Gas accumulation and DML measurements

134 Digestion of feedstuffs was quantified relative to control blanks which contained
135 microbial inoculum, culture medium and reducing agent but no feed sample. Gas
136 production was recorded at 2, 5, 8, 11, 14, 17, 20, 23, 26, 30, 34, 37.5, 44.5, 52, 59, 72,

96.5, 122 and 140 hours after inoculation using the manual pressure transducer technique (PTT) of Theodorou et al. (1994). After the final reading a small aliquot of the culture fluid was acidified for volatile fatty acid (VFA) analysis as described below and bottles were then refrigerated at 4°C. Thereafter, culture fluid was separated from residual plant particles and adherent microbial biomass by filtration through pre-weighed sintered glass crucibles (porosity 1) under reduced pressure. The residue was rinsed with two volumes (ca. 20 ml) of distilled water and oven dried (60 °C) to constant weight for the determination of residual DM. Given that the feed residues included microbial biomass adhering to the residue, the calculation of initial minus residual DM is referred to as DM loss (DML).

Volatile fatty acid (VFA) analysis

Acetic, butyric and propionic acids were measured by gas chromatography at the end of the incubation. One ml of culture fluid was acidified with 5 μl of orthophosphoric acid and stored at 4 °C until analysis. Prior to analysis, acidified samples were defrosted and centrifuged at 3500 g for three minutes. Internal standard (0.2 ml; 15 mM 2-methyl-valeric acid in 0.15 M orthophosphoric acid) was added to the supernatant. A calibration mixture consisting of the following VFA; 15.0 mM acetic acid, 5.0 mM propionic acid, 0.2 mM iso-butyric acid, 2.0 mM n-butyric acid, 0.2 mM iso-valeric acid, and 2.0 mM n-valeric acid in 0.15 M orthophosphoric acid was used as the external standard. VFA quantification was carried out using a Chrompack 9000 chromatograph fitted with an automatic sampler (Chrompack 911) and linked to an IBM PC with Chrompack Mosaic integration software. A WCOT fused silica capillary column (25 m x 32 mm internal

160 diameter) coated with FFAP-CB was used in conjunction with the following
161 chromatographic conditions; injector and detector temperatures were 240 °C and 260 °C,
162 respectively. The column was run isothermally at 155 °C for 8 min and the temperature
163 then increased at a rate of 30 °C min^{-1} to 200 °C. The carrier gas (helium) arrived at the
164 head of the column with a pressure of 75 kPa and a split flow rate of 200 ml min^{-1}. VFA
165 parameters are quantified as total VFA (TVFA; mM), and acetate (Ac), propionate (Pr)
166 and butyrate (Bu) molar proportions (mmol mol^{-1}).
167
168 Statistics and curve fitting
169 Gas accumulation volumes were corrected according to the recorded pressure and the
170 mean control blank volumes were then subtracted from the gas production profiles (as
171 described by Theodorou et al., 1994). Cumulative gas production profiles were described
172 according to the model of France et al. (1993) given below:
173
174 $$y = A - BQ^{t}Z^{\sqrt{t}} \quad (1)$$
175
176 where $Q = e^{-b}$, $Z = e^{-c}$, and $B = Ae^{bT+c\sqrt{T}}$. Here, y denotes cumulative gas production (ml),
177 t is incubation time (h), A is the (predicted) asymptotic value for gas pool size (ml), T is
178 the lag time (h) and b (h^{-1}) and c ($h^{-0.5}$) are rate constants. The equation was fitted to the
179 corrected gas accumulation data using the maximum likelihood programme (MLP; Ross,
180 1980). The time dependent fractional rate of gas production (FRGP; h^{-1}) was calculated
181 using the following equation;
182

183 $$FRGP = b + c/(2\sqrt{t_{50}}) \quad (2)$$

184

185 where b and c are as described above and t_{50} is the time taken to produce 50% of the total
186 gas production (h).

187

188 Data were analysed using Genstat 5 (Lawes Agricultural Trust, 1993). Simple linear and
189 quadratic equations for DMIV and DE on each individual variable were obtained. The
190 variables were arranged into sub-groups as follows; (1) cumulative gas volumes at 5 (g5),
191 11 (g11), 23 (g23), 44.5 (g44), 72 (g72), 96.5 (g96) and 140 (g140) hours following
192 incubation; (2) fitted and derived gas production parameters (as described above with the
193 addition of t_{95}; the time taken to produce 95 % of the total gas production); (3) VFA
194 parameters (as described above) and (4) DML (g kg^{-1}). Stepwise multiple linear
195 regression (SMLR) analysis was also carried out to derive prediction equations from sub-
196 groups of individual variables with and without the addition of DML, i.e: (i) cumulative
197 gas volumes; (ii) fitted and derived gas production parameters; (iii) VFA parameters and
198 (iv) all available parameters. Equations were constrained to allow a maximum of three
199 variables plus a constant in order to avoid problems of overfitting.

200

201 Residual variation for each prediction equation was partitioned into three components
202 using mean-square prediction error (MSPE) analysis (Theil, 1966; Bibby & Toutenburg,
203 1977) as follows:

204

205 $$MSPE = (O - P)^2 + S_P^2 (1 - m)^2 + S_O^2 (1 - r^2) \quad (3)$$

where O and P are the respective means of the actual and predicted values, S_O^2 and S_P^2 are their respective variances, m is the slope of the regression line of O on P and r is their correlation coefficient. The three components are thus due to (1) mean bias (P - O), (2) line bias; the deviation of the slope (m) of the regression of O on P from unity, and (3) the random variation about this regression line. A positive mean bias indicates that the model is generally over predicting relative to the actual values and vice versa while a large line bias is indicative of underlying inadequacies in the structure of the model. If the slope of the regression of O on P is less than unity the model tends to under predict at low actual values and to over predict at high actual values and vice versa. Results are presented in terms of the proportional contribution of each of these three components to the MSPE. The square root of the MSPE is the mean prediction error (MPE) and is reported as a proportion of the mean actual DMIV or DE observed in vivo.

Results

Gas accumulation, VFA production and DML

A wide range of gas accumulation profiles between the feedstuffs were observed (Figures 1a and b). The mean, minimum and maximum values for the modelled gas production parameters along with their SD are shown in Table 2. The lag time, T (h), encountered prior to active fermentation varied with the different feeds. However, the lag time for some feeds may be an overestimate, particularly where starch contents are high resulting in enhanced lactate and propionate production without the concomitant production of gas and hence the appearance of an apparently long lag time (Beuvink & Spoelstra, 1992;

229 Russell & Wallace, 1997). In all but two feeds more than 50 % of the total gas production
230 (A), was produced within the first 35 h of the incubation. In the two exceptions, OS and
231 WS, 50 h of incubation was required to produce ca. 50 % of A. Incubation of OF
232 produced the least gas (127.8 ml) whilst SH produced most gas (242.0 ml) during the 140
233 h incubation period.
234
235 **Figure 1 here**
236
237 Values for the range in total concentration (mM) of TVFA measured at the end of
238 incubations are also shown in Table 2 along with values for Ac, Pr and Bu (mmol mol^{-1}).
239 Production of TVFA was highest after incubation of SH (59.8 mM) and lowest after
240 incubation of WS (29.9 mM). The molar proportions of individual acids varied
241 considerably between feeds with values ranging from 514 (RNO) to 663 (L3); 276 (SB2)
242 to 417 (RNO) and 48 (WS) to 122 (SBM) mmol mol^{-1} for Ac, Pr and Bu respectively.
243 DML values (Table 2) were lowest following incubation of the lucerne sample L2 (382 g
244 kg^{-1}) and highest following incubation of SBM (966 g kg^{-1}).
245
246 **Table 2 here**
247
248 Correlations between predictive parameters
249 There were many significant correlations between the predictive parameters (Table 3).
250 Cumulative gas volumes greater than g5 were all positively correlated as might be
251 expected from the summative nature of these measurements. Positive correlations

252 between A and most of the cumulative gas volumes, Z and FRGP were also seen. t_{50} was
253 positively correlated with t_{95} whilst both t_{50} and t_{95} were negatively correlated with most
254 of the cumulative gas volumes and FRGP. Q was negatively correlated with both Z and
255 FRGP, whilst Z was positively correlated with FRGP. Again, high degrees of correlation
256 were expected between these parameters since both Q and Z are fitted parameters which
257 quantify the rate of gas production whilst FRGP is derived from the negative logarithms of
258 Q and Z. TVFA was positively correlated with cumulative gas volumes greater than g5,
259 B, A and Bu, but not Ac or Pr. Ac and Pr were negatively correlated indicating that
260 variation in fermentation pattern between feeds was mainly expressed by changes in the
261 Ac to Pr ratio. DML was positively correlated with cumulative gas volumes greater than
262 g5, A, FRGP, TVFA and Bu, but negatively correlated with Ac.
263
264 **Table 3 here**
265
266 Prediction equations
267 Significant ($P<0.05$) linear and quadratic relationships between the individual parameters
268 used to predict DMIV and DE are shown in Tables 4 and 5 respectively. Despite
269 statistical significance, prediction of both DMIV and DE (Figure 2a) from DML alone was
270 poor indicating the limitations of predicting in vivo parameters from end point in vitro
271 digestion techniques. g44 was the best single predictor for DMIV in both the linear and
272 quadratic forms with R^2 values of 0.751 and 0.752 respectively. Similarily, g44 was also
273 the best linear and quadratic predictor of DE (Figure 2b) with R^2 values of 0.704 and

0.682 respectively. Individual parameters g72, g96, Q and FRGP also predicted both DMIV and DE with R^2 values greater than 0.6.

Tables 4 and 5 here

Highly significant ($P<0.001$) multiple linear regression equations using the individual subgroups of parameters and all available parameters are shown in Tables 6 and 7 for DMIV and DE respectively. Using cumulative gas production volumes alone to predict DMIV (equations 4 and 5) and DE (equation 15) resulted in predictive equations with higher R^2 and lower RSD values than any of the linear or quadratic equations given in Tables 4 and 5. Including DML with cumulative gas production volumes improved the prediction of DE (equation 16), whilst the prediction of DMIV was not improved when DML was incorporated (equation 6).

Using fitted and derived gas production parameters alone to predict both DMIV and DE was generally less accurate than using the cumulative gas volumes alone (equations 7 & 8 for DMIV and equation 17 for DE). Including DML as a variable in the predictive equation improved the prediction of DMIV (equation 9 & 10) compared with the use of fitted and derived gas production parameters alone (equation 8). The prediction of DE was also improved by the inclusion of DML (equation 18 & 19; Figure 2c) compared to the use of fitted and derived gas production parameters alone (equation 17).

296 Prediction equations with the highest R^2 and lowest RSD values for both DMIV (equation
297 11) and DE (equation 21) were obtained from VFA parameters alone. Incorporating DML
298 as a predictor along with VFA parameters resulted in equations with lower R^2 and higher
299 RSD values (equations 12 and 22, for DMIV and DE respectively) compared to the best
300 equations containing VFA parameters alone.
301
302 When all parameters were made available to the SMLR procedure equations 11 and 21
303 proved to be the best predictors of DMIV and DE respectively in terms of R^2 and RSD
304 values. However, additional equations incorporating cumulative gas volumes, fitted and
305 derived gas production parameters, VFA parameters and DML are also shown in Tables 6
306 and 7 as examples of alternative prediction relationships.
307
308 **Tables 6 and 7 here.**
309
310 The mean predicted DMIV and DE and their standard errors for equations 4 - 26 are
311 shown in Table 8 together with the MSPE, the proportion of MSPE attributable to mean
312 bias, line bias and random error. Also shown is the actual mean bias for each equation
313 and the MPE expressed as a proportion of the mean observed DMIV or DE. Random
314 error accounted for more than 0.99 of the MSPE in all equations except 21, 22, 25 & 26.
315 The actual predictive bias was negligible for the majority of DMIV equations; the
316 exceptions were a small positive bias of 2.0 g kg^{-1} for equation 11 and a small negative
317 bias of 2.5, 5.0 and 2.1 g kg^{-1} for equations 6, 12 and 13 respectively. For the prediction
318 of DE actual predictive bias was also negligible for the majority of predictive equations

with the exceptions of equations 22, 23 and 25, which had positive bias of 1.04, 0.04 and 0.26 MJ kg^{-1} DM, respectively and equations 21 and 26 which showed negative bias of 0.36 and 0.24 MJ kg^{-1} DM respectively. MPE ranged from 0.069 (equation 11) to 0.108 (equation 7) for DMIV and from 0.072 (equation 19) to 0.153 (equation 20) for DE.

Table 8 here.

Figure 2 here

Discussion

Use of equine faeces as a source of inoculum

The cumulative gas production profiles of the feeds reflected their wide diversity, demonstrating the potential of the pressure transducer technique to evaluate digestion kinetics of equine feedstuffs. Equine faeces proved to be a suitable source of inoculum for gas production studies producing typical gas production profiles for the sixteen feedstuffs under investigation. Macheboeuf and Jestin (1997) have also utilised equine faeces as a source of microbial inoculum in gas production studies using the Menke and Steingass (1988) technique. They concluded that equine faeces and caecal fluid were equally good as sources of microbial inoculum for prediction of the in vivo organic matter digestibility (OMD) of 52 forages in horses. Similar conclusions regarding the use of equine caecal and faecal sources of inocula have been reported from our own laboratory (Kirkhope and Lowman, 1996; Lowman et al., 1996).

342 Using faeces to replace rumen fluid as a microbial inoculum was first reported by El Shaer
343 et al. (1987) to determine the in vitro digestibility of a number of samples of grass, grass
344 silage, lucerne, barley straw, soyabean meal and rolled barley. They used a version of the
345 two-stage Tilley and Terry technique (1963) and obtained digestibility results in vitro
346 which were closely correlated (R^2 = 0.98) with in vivo apparent digestibilities. The
347 relationship was represented by the equation; in vivo apparent digestibility = in vitro
348 digestibility x 1.003. However, these high correlations were only achieved after the basic
349 Tilley and Terry technique (1963) had been modified according to the nature of the
350 substrate to be fermented; for example, a nitrogen source was added to all feedstuffs while
351 the fermentation time for barley straw was increased from 48 to 72 h. The ability of
352 faeces to replace rumen fluid in Tilley and Terry incubations has also been reported by
353 Omed et al., (1989) and Akhter et al. (1994). In the latter study the results were not
354 compared to in vivo apparent digestibilities but the authors did find a high R^2 value (0.97)
355 for the relationship between in vitro digestibility determined using sheep rumen fluid and
356 in vitro digestibility determined using freshly voided cow faeces. Moreover, the
357 relationship held even after freezing and thawing of the faeces. Using cow faeces in the
358 pressure transducer technique as an alternative to rumen fluid has been investigated by
359 Harris et al. (1995). They suggest that although faecal material is a suitable alternative to
360 rumen liquor, separate calibrations with in vivo data are required as the rumen fluid and
361 faecal inoculum produced slightly different gas production profiles.
362
363 Faeces, as the microbial inoculum for in vitro digestibility studies with equines have
364 several advantages over caecal digesta. Faeces are a cheap, readily available source of

micro-organisms, do not require the use of surgically prepared (fistulated) animals, and can be collected from any individual or several animals thereby minimizing the effects of animal to animal variation. A faecal inoculum may also provide the opportunity of studying fermentation in horses with compromised gut function, such as in animals with chronic diarrhoea.

Relationships between in vitro and in vivo measurements

Numerous in vivo and in vitro techniques are available for studying digestion and the nutritive value of feedstuffs in ruminants (Johnson, 1966). Compared to ruminants, far fewer studies have been conducted with horses yet feed represents approximately 60 to 70% of the cost associated with keeping horses (Cunha, 1980). Increased nutritive demands placed upon equines in relation to reproduction, growth, recreational / sporting activities and draught work means that it is important to feed a balanced ration which will enable the horse to perform to its full potential. Consequently, routine prediction of the nutritive value of feeds is just as important when devising rations for productive equines as when devising rations for productive ruminants.

Routine prediction of equine feed values has been reported using the chemical composition of feedstuffs (Martin-Rosset et al., 1996a); a pepsin cellulase method (Martin-Rosset et al., 1996b) and near infra-red reflectance spectrophotometry (Andrieu et al., 1996 a and b) as predictor variables. These studies derived predictive relationships for 52 forages and produced equations with R^2 values ranging from 0.878 to 0.931. Fonnesbeck, (1981) has also published equations to predict the DE of equine feeds from

388 their chemical composition with R^2 values ranging from 0.754 to 0.800. These equations
389 were used by NRC (1989) to predict the DE values which form part of their compiled
390 tables of nutritive values for equine feeds.
391
392 In the current study the best single predictor of both DMIV and DE was the cumulative
393 gas volume recorded at 44.5 hours incubation (g44). In addition, the SMLR procedure
394 selected g44 as the first parameter in all equations which incorporated cumulative gas
395 volumes as predictor variables (equations 4, 5, 6, 13, 14, 15, 16 and 23). g44 is likely to
396 be a function of both the rate and extent of feed degradation and hence gas production
397 during incubation of feedstuffs. Equations selected from modelled gas production
398 parameters (equations 7, 8, 9, 10, 17, 18 and 19) also contain predictor variables
399 predominantly reflecting the rate and extent of feed degradation (A, FRGP, Q, Z, and t50)
400 although T was also included in equation 19 ($R^2 = 0.878$; RSD = 0.99).
401
402 In a study using caecal fluid as the source of inoculum Macheboeuf et al. (1997) have also
403 reported the usefulness of cumulative gas volumes to predict in vivo parameters. They
404 found that the cumulative gas volume at 24 h incubation in conjunction with feed CP
405 content predicted the % OMD of grass forages and hays with an R^2 of 0.87 and an RSD of
406 2.7. They also reported that cumulative gas volume at 24 h could be used alone to predict
407 OMD of alfalfa hays with an R^2 of 0.76 and an RSD of 2.2. In the same study both the
408 rate and extent of gas production were found to correlate well with OMD in alfalfa hays
409 whilst the rate of gas production alone predicted OMD in grass forages and hays with an
410 R^2 of 0.74 and an RSD of 3.7. Similar findings were reported when equine faeces was

411 used as the source of microbial inoculum during gas production studies with the same
412 group of forages (Macheboeuf and Jestin, 1997). Following incubation of ten hays with
413 rumen fluid, Khazaal et al. (1993) reported significant relationships between both the rate
414 and extent of gas production in vitro and apparent DM digestibility in vivo determined in
415 sheep.
416
417 Of the multiple linear regression equations derived in this study, those incorporating only
418 VFA parameters as predictor variables had the highest R^2 and lowest RSD's for both
419 DMIV (equation 11; $R^2 = 0.859$; RSD = 51.2) and DE (equation 21; $R^2 = 0.865$; RSD =
420 1.04). TVFA is likely to be a function of the extent of feed degradation whilst variations
421 in Ac and Pr proportions probably reflect the starch and fibre contents of the individual
422 feedstuffs. The use of VFA to predict the metabolisable energy content of ruminant feeds
423 has been proposed by Dennison and Phillips (1983). They reported that cellulose
424 digestion and VFA production were highly correlated (r = 0.88 - 0.99). The equations
425 derived in the current study from VFA parameters measured following incubation of a
426 wide range of equine feedstuffs support their argument that VFA produced in batch
427 culture in vitro systems should provide a measure of the energy value of feeds.
428
429 Although DML as a single predictor gave poor estimations of in vivo values, inclusion of
430 DML in multiple regression equations did improve prediction of DE when included as a
431 term with cumulative gas volumes (equation 16; $R^2 = 0.799$; RSD = 1.27) and modelled
432 gas production parameters (equation 19; $R^2 = 0.878$; RSD = 0.99). However, in practical
433 terms the determination of DML through filtration is time consuming and can cause

problems when feedstuffs agglutinate during the incubation leading to poor filtration characteristics and thus highly variable results. Another potential source of error in DML measurements is the attatchment of micro-organisms to feed particles leading to under estimation of DML (Theodorou et al., 1995).

When all parameters were made available to the SMLR procedure, equations derived from sub-groupings of predictor variables were confirmed as having the highest R^2 and lowest RSD's (equations 5 and 11 for DMIV and equations 18, 19 and 21 for DE). Although additional highly significant relationships could be found (equations 13, 14, 23, 24, 25 and 26) they generally did not improve the accuracy of either DMIV or DE prediction in terms of R^2 and RSD. However, MSPE analysis identified systematic bias in equations 11, 13, 21, 23, 25 and 26 making them unsuitable for routine use. The identification of bias in equations 11 and 21 demonstrate that although these were the equations with the highest R^2 and lowest RSD's for DMIV and DE respectively, their use in practice would lead to large prediction errors. This finding demonstrates the disadvantages of selecting predictive equations on the basis of their R^2 and RSD values alone. MSPE analysis has proved a useful statistical method for assessing the reliability of equations derived by SMLR. The absence of either systematic or line bias in the remaining equations indicates the potential of these predictive relationships to describe in vivo parameters for a diverse range of equine feedstuffs. In particular, equation 24 (Figure 2d) had a high R^2, low RSD, negligible bias and a small MPE ($R^2 = 0.852$; RSD = 1.09; bias = <0.01; MPE = 0.08).

456 The predictor variables used in this study increased in complexity from the cumulative gas
457 volumes which require only the quantative measurement of gas; through modelling gas
458 production parameters which requires sophisticated mathematical analysis of data (France
459 et al., 1993); to measuring DML and VFA production requiring filtration and gas
460 chromatography (GC) respectively. The range of analytical methodologies utilised in this
461 evaluation therefore lend themselves to various experimental environments ranging from
462 the simplest research station to more sophisticated laboratories. Potential disadvantages
463 associated with the use of complicated analytical equipment such as GC's are the increase
464 in time, labour input and cost involved in routine feed analysis. One of the original aims
465 of this in vitro gas production technique (Theodorou et al., 1994) was to provide a simple
466 analytical tool for feed evaluation studies which could be employed in a range of
467 laboratories world-wide. This study has confirmed the suitability of simple cumulative
468 gas volumes or modelled gas production parameters as predictors of in vivo nutritive feed
469 values for equines and demonstrated the widespread applicability of this in vitro batch
470 culture technique for routine feed evaluation.
471
472 Concluding remarks
473 The prediction equations developed in this study correlated well with in vivo apparent
474 digestibility and DE measurements demonstrating that the pressure transducer technique
475 along with associated DML and VFA analysis has considerable potential as a routine
476 predictor of nutritive value for a wide range of equine feeds. However, multiple linear
477 regression equations of the type devised in this study may be unstable as predictive
478 models when used with independent data sets due to multi-collinearity among predictor

479 variables. For example, equation 15 contains three highly correlated cumulative gas
480 volumes as predictors. In addition, the relatively small number of feeds (16) used in this
481 study represents a major limitation when assessing the wider applicability of the predictive
482 relationships derived.
483
484 Future development of routine prediction equations for equine feeds should ideally focus
485 on 1) greatly increasing the size of the in vivo database; 2) grouping of feeds into similar
486 feed classes ie: at least forages and concentrates (Menke and Steingass, 1988) and 3)
487 utilising more robust statistical methods such as principal component, ridge regression or
488 partial least squares analysis to devise relationships, thereby improving the accuracy and
489 reliability of the derived prediction equations.
490
491
492
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497
498

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Table 1. Distribution of chemical composition, dry matter digestibility in vivo (DMIV) and digestible energy content (DE) of sixteen equine feeds (g kg^{-1} DM unless otherwise stated).

	Mean	Minimum	Maximum	SD
Chemical composition				
DM (g kg^{-1})	873	681	969	62.7
OM	946	884	983	30.9
CP	227	37	519	191.3
ADF	239	39	509	168.9
NDF	364	78	762	247.4
Starch	201	0	630	245.9
GE (MJ kg^{-1} DM)	18.8	17.2	19.9	0.67
In vivo values				
DMIV (g kg^{-1})	665	443	920	136.3
DE (MJ kg^{-1} DM)	12.3	7.7	17.7	2.83

For abbreviations see text.

Table 2. Distribution of cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss values for sixteen equine feeds.

	Mean	Minimum	Maximum	SD
Cumulative gas volumes				
g5	7.6	1.5	17.8	5.24
g11	31.2	8.6	48.6	12.60
g23	83.0	27.2	127.1	31.39
g44	139.1	69.1	205.0	43.55
g72	163.0	102.1	219.1	40.32
g96	172.9	115.4	229.1	37.24
g140	184.0	131.2	239.9	34.75
Fitted and derived gas production parameters				
Q	0.9434	0.8800	0.9740	0.0307
Z	1.234	0.939	1.923	0.3004
B	158.3	77.2	267.0	47.30
A	181.8	127.8	242.0	32.49
t50	27.2	18.5	49.7	9.48
t95	84.1	51.2	155.2	30.59
T	4.15	1.04	7.58	1.770
FRGP	0.0402	0.0197	0.0622	0.01318
Volatile fatty acid parameters				
TVFA	43.2	29.9	59.8	9.59
Ac	603	514	663	42.1
Pr	326	276	417	38.3
Bu	71	48	122	23.0
Dry matter loss				
DML	693.5	382.0	966.0	198.02

For abbreviations see text.

Table 3. Correlation matrix for cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss following incubation of sixteen equine feeds.

g11	0.470																		
g23	0.279	0.958																	
g44	0.006	0.733	0.861																
g72	-0.019	0.653	0.788	0.974															
g96	-0.063	0.594	0.742	0.949	0.993														
g140	-0.110	0.560	0.719	0.932	0.983	0.994													
Q	0.183	-0.334	-0.492	-0.808	-0.750	-0.730	-0.711												
Z	-0.319	-0.017	0.146	0.577	0.550	0.547	0.536	-0.928											
B	0.193	0.533	0.466	0.132	0.163	0.159	0.174	0.453	-0.687										
A	-0.137	0.496	0.652	0.894	0.965	0.979	0.992	-0.687	0.543	0.149									
t50	-0.419	-0.903	-0.893	-0.714	-0.606	-0.542	-0.482	0.403	-0.087	-0.373	-0.390								
t95	-0.320	-0.798	-0.834	-0.852	-0.773	-0.726	-0.670	0.680	-0.426	-0.106	-0.596	0.881							
T	-0.835	-0.230	-0.031	0.336	0.326	0.355	0.373	0.573	-0.658	-0.309	0.375	0.124	-0.063						
FRGP	0.015	0.720	0.830	0.925	0.825	0.783	0.753	-0.867	0.630	-0.020	0.689	-0.758	-0.873	0.388					
TVFA	-0.123	0.582	0.652	0.665	0.740	0.756	0.762	-0.209	-0.007	0.645	0.750	-0.466	-0.460	0.190	0.440				
Ac	0.526	-0.077	-0.231	-0.593	-0.574	-0.560	-0.574	0.780	-0.812	0.412	-0.601	0.074	0.342	-0.651	-0.556	-0.239			
Pr	-0.284	-0.133	-0.016	0.358	0.376	0.378	0.403	-0.654	0.778	-0.634	0.461	0.195	-0.111	0.371	0.292	-0.074	-0.838		
Bu	-0.487	0.357	0.443	0.483	0.417	0.388	0.373	-0.336	0.191	0.294	0.325	-0.457	-0.441	0.566	0.526	0.551	-0.434	-0.128	
DML	-0.399	0.499	0.599	0.700	0.706	0.707	0.713	-0.455	0.282	0.341	0.706	-0.398	-0.445	0.483	0.583	0.831	-0.616	0.264	0.679
	g5	g11	g23	g44	g72	g96	g140	Q	Z	B	A	t50	t95	T	FRGP	TVFA	Ac	Pr	Bu

Coefficients > 0.497 are significant ($P < 0.05$). For abbreviations see text.

Table 4. Linear and quadratic relationships between dry matter digestibility in vivo (DMIV) and cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss.

		Linear $y = a + bx$					Quadratic $y = a + bx + cx^2$				
x	a	b	R^2	RSD	Significance	a	b	c	R^2	RSD	Significance
g5											
g11	458.8	6.610	0.328	112	*	334	17.80	-0.201	0.325	112	*
g23	420.1	2.949	0.423	104	**	219	9.11	-0.0397	0.467	99.5	**
g44	283.5	2.743	0.751	68	***	104	5.66	-0.0107	0.752	67.9	***
g72	194.9	2.884	0.708	73.6	***	-256	8.85	-0.0186	0.727	71.3	***
g96	141.5	3.027	0.661	79.4	***	-558	11.56	-0.0249	0.688	76.2	***
g140	102	3.060	0.580	88.3	***	-1270	18.33	-0.0411	0.645	81.2	***
Q	4017	-3533	0.616	84.5	***	-27719	64828	-36790	0.632	82.7	***
Z	305	291.4	0.370	108	**	335	247	16	0.322	112	*
B											
A	89	3.168	0.537	92.7	***	-1008	15.34	-0.0328	0.568	89.6	**
t50	918.6	-9.330	0.379	107	**	794	-1.0	-0.124	0.338	111	*
t95	959.8	-3.504	0.591	87.2	***	1105	-6.75	0.0161	0.574	89	**
T											
FRGP	317.4	8634	0.675	77.7	***	111	19925	-138837	0.676	77.6	***
TVFA	327	7.820	0.253	118	*	-1454	91.8	-0.946	0.473	99	**
Ac	1814	-1.907	0.300	114	*	13935	-43.3	0.0351	0.500	96.4	**
Pr						5065	-27.5	0.0423	0.349	110	*
Bu											
DML	361	0.439	0.364	109	**	597	-0.28	0.00051	0.326	112	*

For abbreviations see text.

Table 5. Linear and quadratic relationships between <u>in vivo</u> digestible energy content (DE) and cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss.

		Linear	$y = a + bx$				Quadratic	$y = a + bx + cx^2$			
x	a	b	R^2	RSD	Significance	a	b	c	R^2	RSD	Significance
g5											
g11	8.73	0.1137	0.203	2.53	*						
g23	7.75	0.0545	0.319	2.34	*	4.34	0.1590	-0.000675	0.326	2.32	*
g44	4.58	0.05532	0.704	1.54	***	4.84	0.0510	0.000016	0.682	1.60	***
g72	2.77	0.0583	0.668	1.63	***	-0.32	0.099	-0.000127	0.647	1.68	***
g96	1.61	0.0617	0.634	1.71	***	-4.9	0.141	-0.000231	0.616	1.75	***
g140	0.70	0.0629	0.567	1.86	***	-17.1	0.261	-0.000532	0.571	1.85	**
Q	83.8	-75.8	0.654	1.67	***	-105	331	-219	0.631	1.72	***
Z	4.07	6.65	0.461	2.08	**	6.5	2.9	1.30	0.421	2.15	*
B											
A	0.32	0.0658	0.537	1.93	***	-14.2	0.227	-0.000434	0.528	1.94	**
t50	16.82	-0.1673	0.264	2.43	*						
t95	18.11	-0.0693	0.530	1.94	***	21.96	-0.155	0.000426	0.517	1.97	**
T											
FRGP	5.35	172.0	0.615	1.76	***	5.04	189	-211	0.586	1.82	***
TVFA	5.32	0.1609	0.247	2.46	*	-26.4	1.658	-0.01686	0.393	2.21	*
Ac	42.08	-0.0494	0.508	1.99	***	224.8	-0.673	0.000529	0.604	1.78	***
Pr	-0.37	0.0387	0.223	2.50	*	95.8	-0.531	0.000833	0.441	2.12	**
Bu											
DML	5.11	0.01033	0.487	2.03	**	10.70	-0.0068	0.0000121	0.463	2.08	**

For abbreviations see text.

Table 6. Prediction of dry matter digestibility in vivo (DMIV) from cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss.

Parameters	Equation no.	Prediction equation	R^2	RSD
Cumulative gas volumes only	4	y = -1039 + 349 log g44	0.771	65.3
	5	y = 234.2 + 4.907 g44 - 8.15 g23 + 13.63 g11	0.836	55.3
	6	y = -1539 - 1.87 g23 + 0.061 DML + 474 log g44	0.791	62.4
Fitted and derived gas production	7	y = 1746 + 331 log FRGP	0.705	74.1
parameters	8	y = 155 + 1.50 A + 6209 FRGP	0.720	72.1
	9	y = 1191 + 1.142 A + 0.065 DML + 238.3 log FRGP	0.733	70.4
	10	y = 1261 + 0.160 DML + 225.4 log FRGP + 159.0 log Z	0.755	67.5
VFA parameters	11	y = 15431 - 53.58 Ac + 0.04426 Ac^2 + 368.1 log TVFA	0.859	51.2
	12	y = 144940 + 0.372 DML + 44.8 Ac - 26807 log Ac	0.663	79.1
All available parameters		Equations 5 and 11 were selected.		
	13	y = 7106 - 25.66 Ac + 0.0211 Ac^2 + 270.9 log g44	0.856	51.8
	14	y = -975 + 0.055 DML + 328.1 log g44	0.757	67.2

For abbreviations see text. All equations shown are significant ($P < 0.001$).

Table 7. Prediction of digestible energy content (DE) from cumulative gas volumes, fitted and derived gas production parameters, volatile fatty acid parameters and dry matter loss.

Parameters	Equation no.	Prediction equation	R^2	RSD
Cumulative gas volumes	15	y = -2.65 + 0.0941 g44 - 0.1112 g23 + 3.32 log g11	0.788	1.30
	16	y = -32.91 - 0.0543 g23 + 0.00429 DML + 9.57 log g44	0.799	1.27
Fitted and derived gas production parameters	17	y = 4.36 + 0.0295 A + 4.60 Z - 0.1152 t50	0.768	1.36
	18	y = 4.78 + 0.00629 DML - 53.3 log Q	0.803	1.26
	19	y = -0.68 + 0.01087 DML + 6.82 Z - 2.297 log T	0.878	0.99
VFA parameters	20	y = 200.9 - 29.48 log Ac	0.527	1.95
	21	y = 251.4 - 0.857 Ac + 0.000692 Ac^2 + 6.55 log TVFA	0.865	1.04
	22	y = 2298 + 0.00727 DML + 0.692 Ac - 423 log Ac	0.761	1.38
All available parameters		Equations 18, 19 and 21 were selected.		
	23	y = 103 - 0.1209 g44 + 19.13 log g44 - 26.14 log Ac	0.856	1.08
	24	y = 290 - 493 FRGP + 21.87 log FRGP - 29.13 log Ac	0.852	1.09
	25	y = 201.7 + 0.0419 A - 0.64 Ac + 0.000518 Ac^2	0.754	1.40
	26	y = 744 + 0.0478 A + 0.478 Pr - 155.1 log Pr	0.689	1.58

For abbreviations see text. All equations shown are significant ($P < 0.001$).

Table 8. Prediction precision of equations 4 - 14 for predicting dry matter digestibility in vivo (DMIV) and for equations 15 - 26 for predicting the digestible energy (DE) content of sixteen equine feedstuffs.

		Predicted			Proportion of MSPE				
Equation no[§]		Mean	s.e.	MSPE[†]	Bias	Line	Random	Bias[‡]	MPE
DMIV	4	665	30.2	3596	<0.001	<0.001	0.999	<0.1	0.090
	5	665	31.8	2443	<0.001	<0.001	0.999	<0.1	0.074
	6	662	31.1	3118	0.002	<0.001	0.998	-2.5	0.084
	7	665	29.0	5131	<0.001	<0.001	0.999	<0.1	0.108
	8	678	30.1	4512	<0.001	<0.001	0.999	<0.1	0.101
	9	665	30.2	3969	<0.001	<0.001	0.999	<0.1	0.095
	10	665	30.5	3647	<0.001	<0.001	0.999	<0.1	0.091
	11	667	32.1	2099	0.002	<0.001	0.998	2.0	0.069
	12	660	29.2	5039	0.005	<0.001	0.995	-5.0	0.107
	13	663	32.1	2151	0.002	<0.001	0.998	-2.1	0.070
	14	665	30.3	3911	<0.001	<0.001	0.999	<0.1	0.094
DE	15	12.26	0.644	1.359	<0.001	<0.001	0.999	<0.01	0.095
	16	12.28	0.649	1.289	<0.001	<0.001	0.999	<0.01	0.093
	17	12.27	0.639	1.488	<0.001	<0.001	0.999	<0.01	0.099
	18	12.28	0.645	1.366	<0.001	<0.001	0.999	<0.01	0.095
	19	12.27	0.672	0.783	<0.001	<0.001	0.999	<0.01	0.072
	20	12.24	0.529	3.541	<0.001	<0.001	0.999	<0.01	0.153
	21	11.91	0.674	0.996	0.129	<0.001	0.870	-0.36	0.081
	22	13.31	0.635	2.608	0.413	<0.001	0.586	1.04	0.132
	23	12.31	0.666	0.928	0.002	<0.001	0.997	0.04	0.079
	24	12.28	0.665	0.953	<0.001	<0.001	0.999	<0.01	0.080
	25	12.61	0.629	1.689	0.067	<0.001	0.932	0.26	0.106
	26	11.93	0.614	2.113	0.056	<0.001	0.944	-0.24	0.118

[§] For equation details see Tables 6 and 7.

[†] $(g\ kg^{-1})^2$ for equations 4 - 14 and $(MJ\ kg^{-1})^2$ for equations 15 - 26.

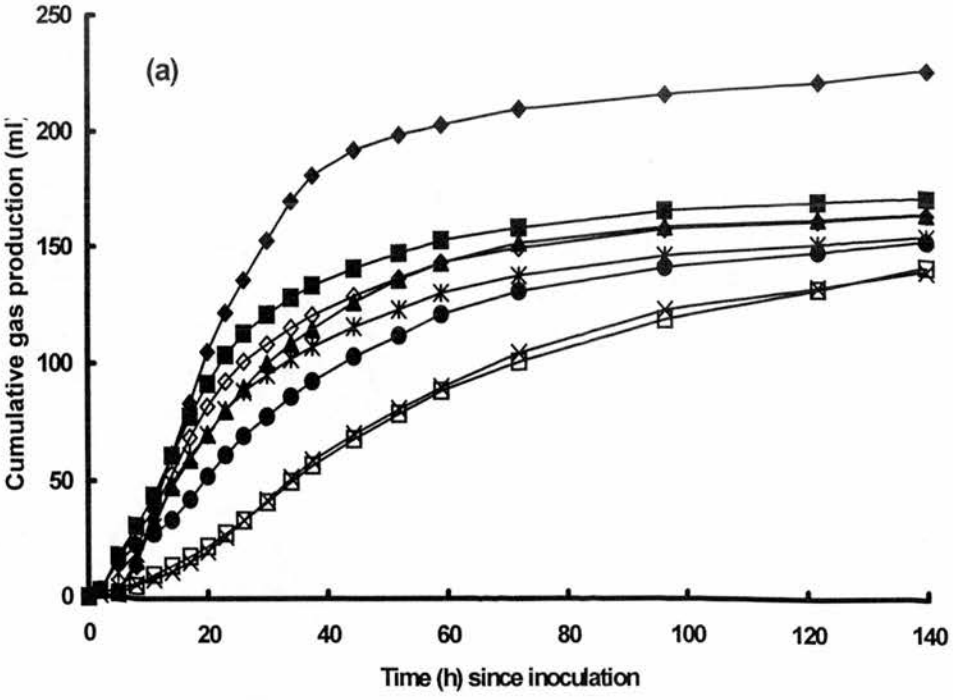
[‡] $g\ kg^{-1}$ for equations 4 - 14 and $MJ\ kg^{-1}$ for equations 15 - 26.

Figure Legends

Figure 1. Fitted cumulative gas production profiles (France et al., 1993) determined with the pressure transducer technique for **(a)** three samples of lucerne (L1, -✳-; L2, -◊-; L3, -■-), oat straw (OS, -□-), wheat straw (WS, -X-), grass haylage (GH, -●-), soyabean meal (SBM, -▲-), barley grain (BG, -◆-) and **(b)** two SBM / BG mixes (SB1, -X- and SB2 -✳-), soya hulls (SH, -◆-), naked oats (NO, -▲-), bruised oats (BO, -●-), rolled naked oats (RNO, -■-), alfalfa / maize (AM, -◊-) and a naked oat / oatfeed mix (OF, -□-) during a 140 h incubation with a microbial inoculum prepared from fresh pony faeces.

Figure 2. The relationship between digestible energy content (DE) and **(a)** dry matter loss in vitro, DML (R^2 = 0.487; RSD = 2.03), **(b)** cumulative gas volume at 44.5 h incubation, g44 (R^2 = 0.704; RSD = 1.54), **(c)** gas production parameters, Z and log T, and DML (equation 19; R^2 = 0.878; RSD = 0.99) and **(d)** FRGP, log FRGP and Ac (equation 24; R^2 = 0.852; RSD = 1.09).

669 **Figure 1**



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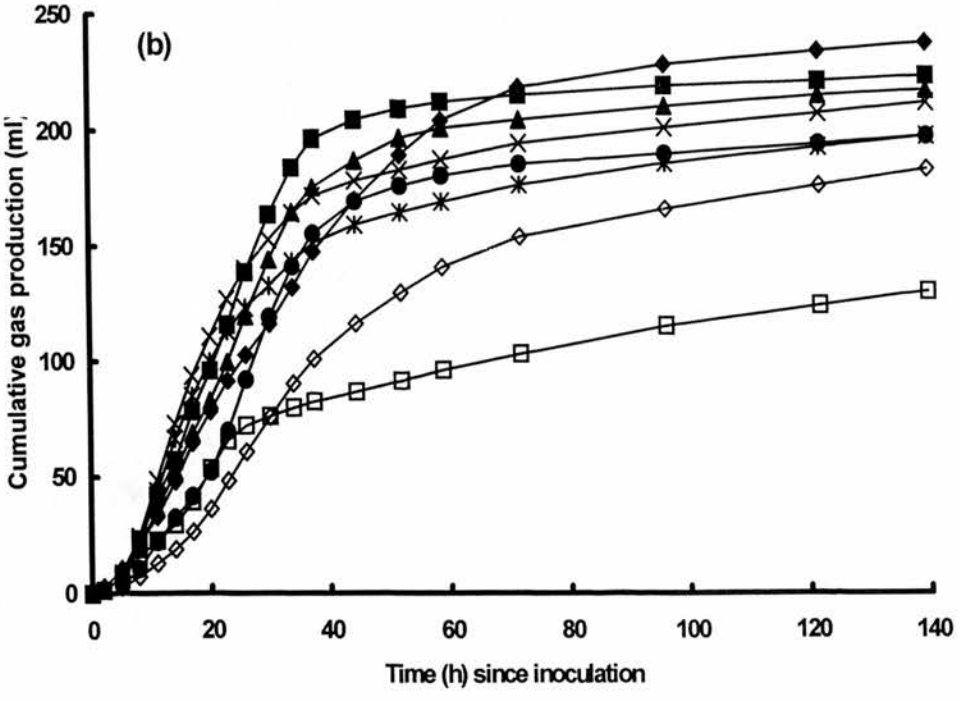

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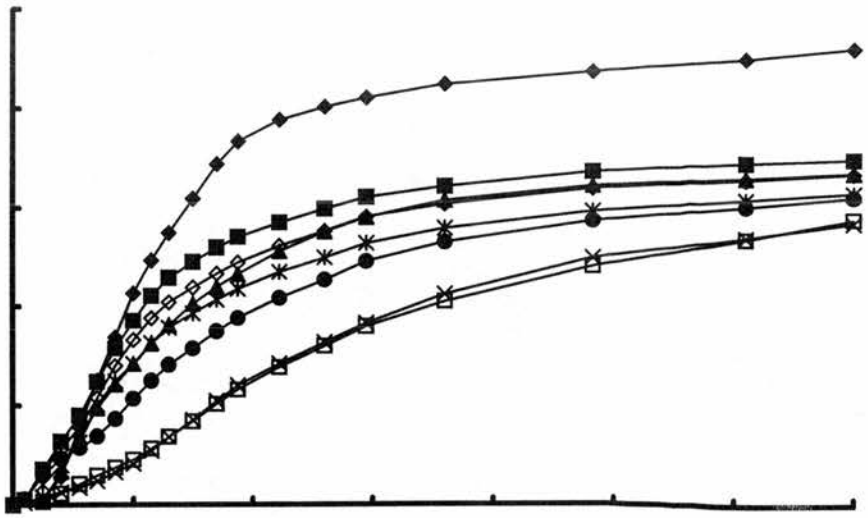

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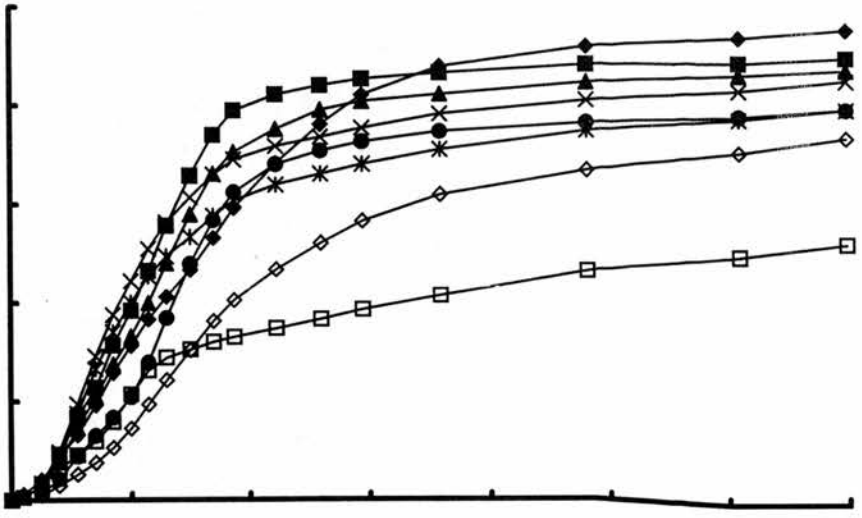

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679 **Figure 2**
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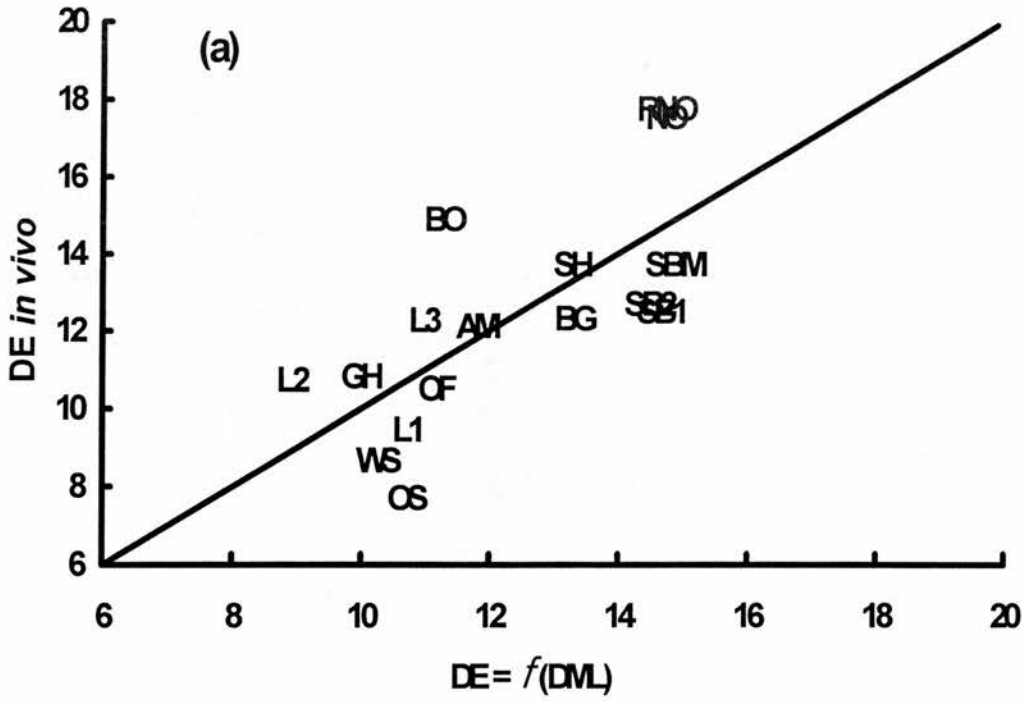

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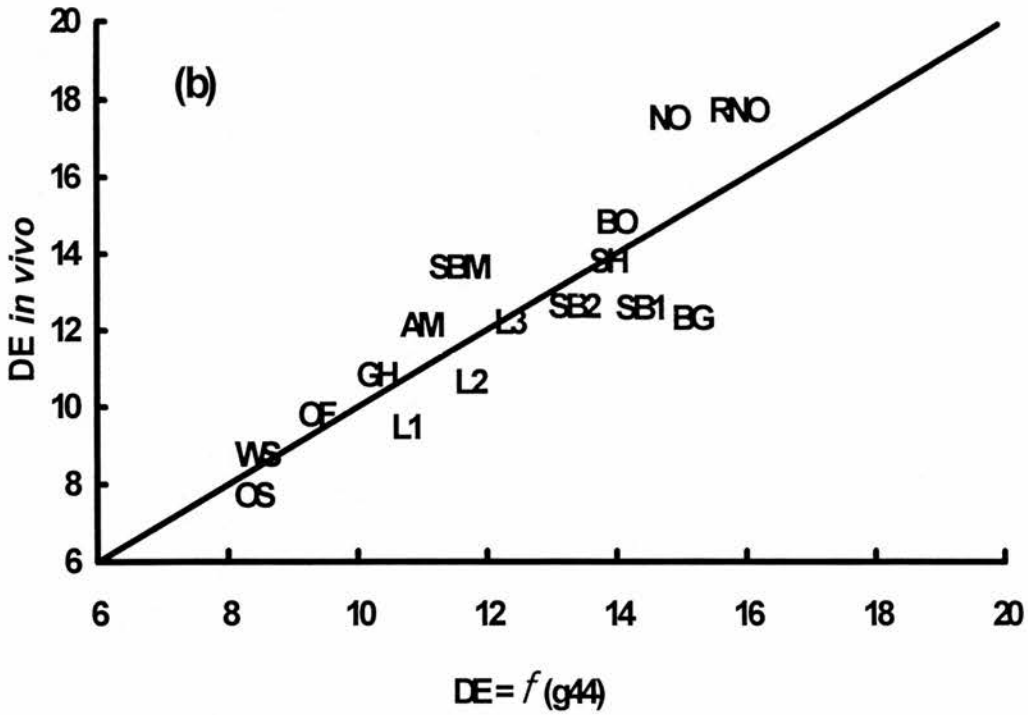

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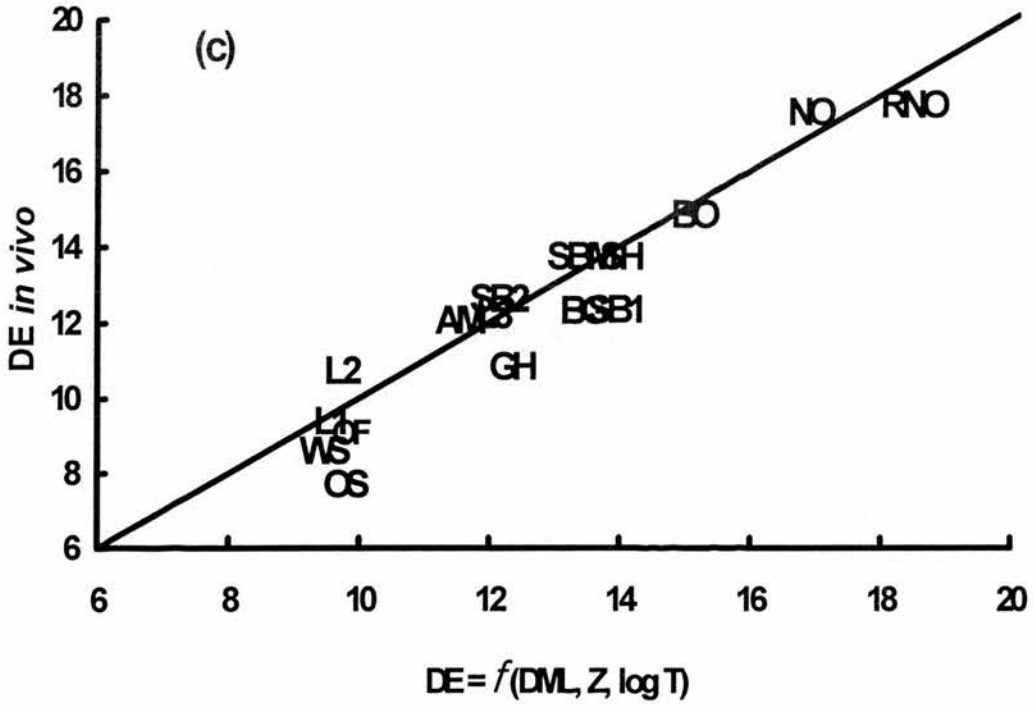

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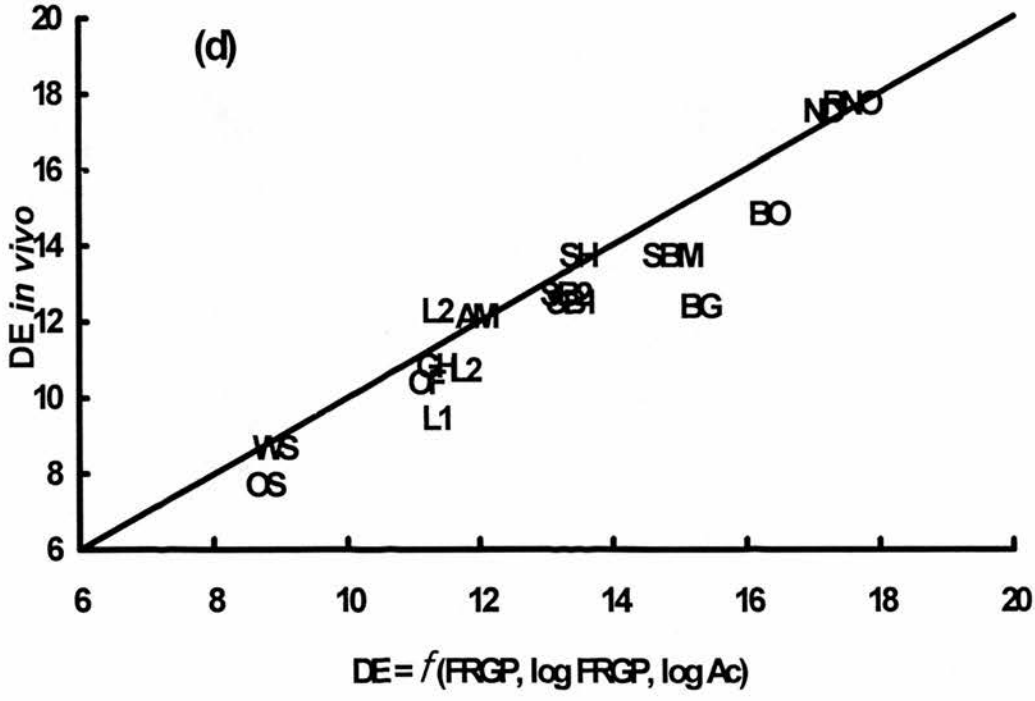

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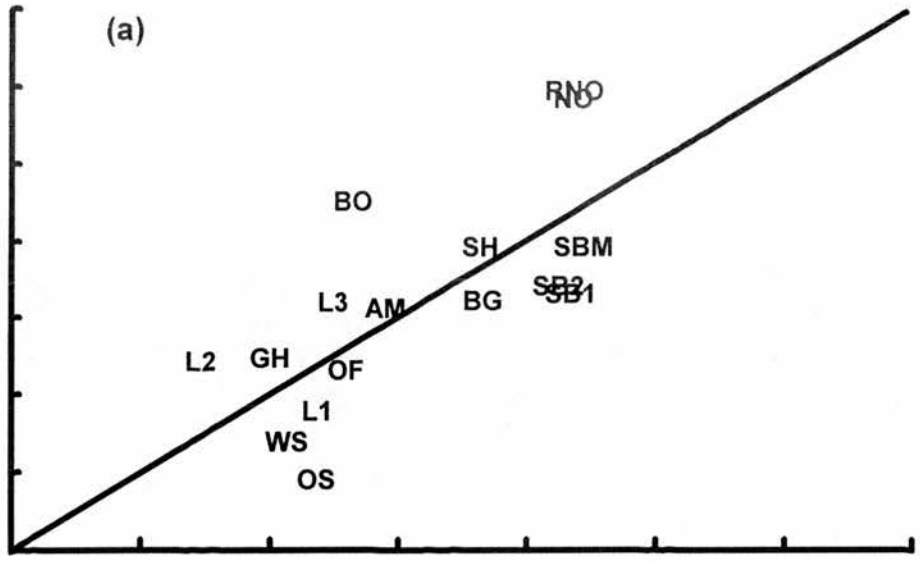

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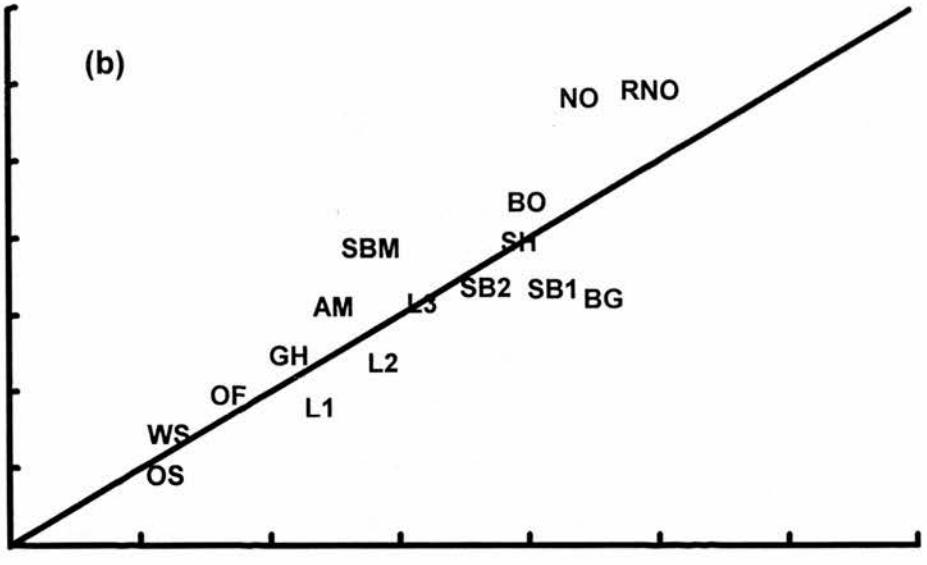

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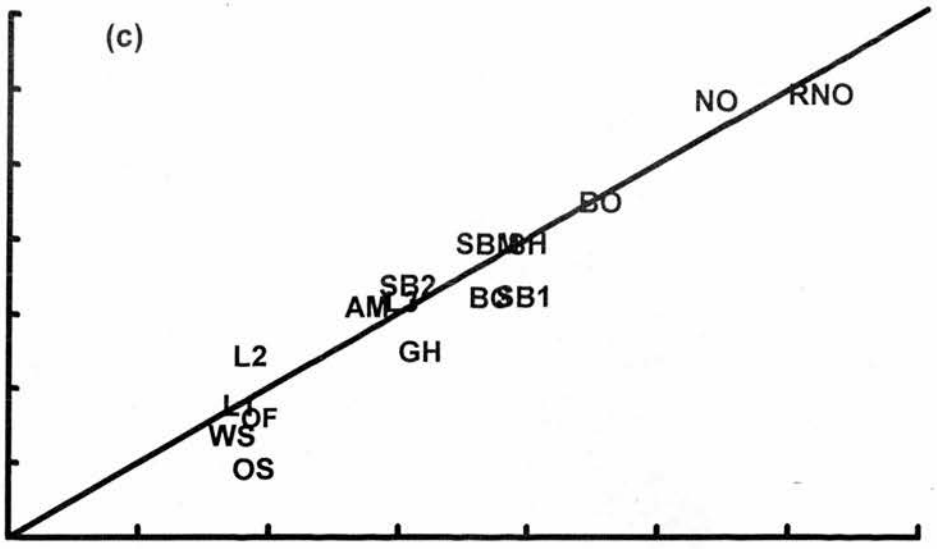
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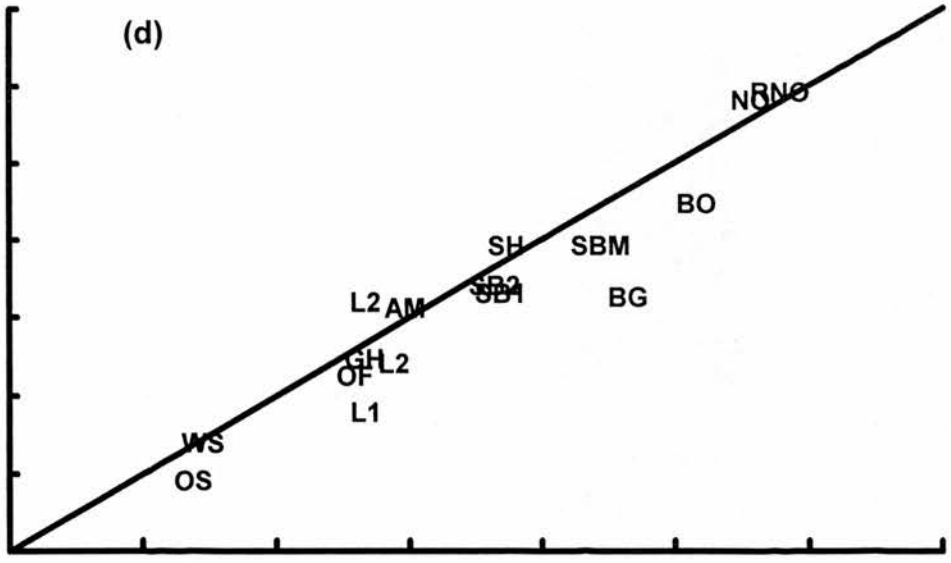
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